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(54) Title: LIPID DERIVATIVES OF ANTIVIRAL NUCLEOSIDES, LIPOSOMAL INCORPORATION AND METHOD OF USE			
(57) Abstract			
<p>Compounds are disclosed for treating AIDS, herpes, and other viral infections by means of lipid derivatives of antiviral agents. The compounds consist of nucleoside analogues having antiviral activity which are linked, commonly through a phosphate group at the 5' position of the pentose residue, to one of a selected group of lipids. The lipophilic nature of these compounds provide advantages over the use of the nucleoside analogue alone. It also makes it possible to incorporate them into the lamellar structure of liposomes, either alone or combined with similar molecules. In the form of liposomes, these antiviral agents are preferentially taken up by macrophages and monocytes, cells which have been found to harbor the target HIV virus. Additional site specificity may be incorporated into the liposomes with the addition of ligands, such as monoclonal antibodies or other peptides of proteins which bind to viral proteins. Effective nucleoside analogues are dideoxynucleosides, azidothymine (AZT), and acyclovir; lipid groups may be glycolipids, sphingolipids, phospholipids or fatty acids. The compounds persist, after intracellular hydrolysis, as phosphorylated or non-phosphorylated antiviral nucleosides. The compounds are effective in improving the efficacy of antiviral nucleoside analogues by prolonging the antiviral activity after the administration of the drug has ended, and in preventing retroviral replication in HIV infections which have become resistant to therapy with conventional forms of the antiretroviral agents.</p>			

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LIPID DERIVATIVES OF ANTIVIRAL NUCLEOSIDES,
LIPOSOMAL INCORPORATION AND METHOD OF USE

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Background of the Invention

The present invention relates generally to the treatment of viral infections using lipid derivatives of antiviral nucleoside analogues. More particularly, the present invention relates to lipid, and especially phospholipid, derivatives of modified antiviral nucleoside analogues which can be integrated into the structure of liposomes, thereby forming a more stable liposomal complex which can deliver greater amounts of drugs to target cells with less toxicity.

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The publications and other reference materials referred to herein are hereby incorporated by reference, and are listed for convenience in the bibliography appended at the end of this specification.

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There has been a great deal of interest in recent years in the use of nucleoside analogues to treat viral infections. A nucleoside consists of a pyrimidine or purine base which is linked to ribose, a five-carbon sugar having a cyclic structure. The antiviral nucleoside analogues closely resemble natural nucleosides and are designed to inhibit viral functions by preventing the synthesis of new DNA or RNA. Nucleosides are enzymatically assembled into DNA or RNA.

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During DNA synthesis, free nucleoside triphosphates (nucleosides with three phosphate groups attached) react with the end of a growing DNA chain. The reaction involves the linking of the phosphate group at the 5' position on

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the incoming nucleoside triphosphate with the hydroxyl group at the 3' position of the sugar ring on the end of the forming DNA chain. The other two phosphate groups are freed during the reaction, thereby resulting in the 5 addition of a nucleotide to the DNA chain.

Nucleoside analogues are compounds which mimic the naturally occurring nucleosides sufficiently so that they are able to participate in viral DNA synthesis. However, the antiviral nucleoside analogues have strategically 10 located differences in chemical structure which inhibit viral enzymes such as reverse transcriptase or which prevent further DNA synthesis once the analogue has been attached to the growing DNA chain.

Dideoxynucleosides are antiviral compounds that lack 15 the hydroxyl groups normally present at the second and third position of ribose. When a dideoxynucleoside is incorporated into a growing DNA chain, the absence of the 3-OH group on its ribose group makes it impossible to attach another nucleotide and the chain is terminated.

20 Dideoxynucleosides are particularly useful in treating retroviral infections where viral replication requires the transcription of viral RNA into DNA by viral reverse transcriptase. Other nucleoside analogues include deoxynucleosides and nucleosides analogues having only a 25 fragment of ribose or other pentose connected to the base molecule.

Acquired immunodeficiency syndrome (AIDS) is caused by 30 the human immunodeficiency virus (HIV). HIV infects cells bearing the CD4 (T4) surface antigen, such as CD4+ helper lymphocytes, CD4+ monocytes and macrophages and certain other CD4+ cell types. The HIV infection of CD4+ lymphocytes results in cytolysis and cell death which contributes to the immunodeficiency of AIDS; however, CD4+ monocytes and macrophages may not be greatly harmed by the 35 virus. Viral replication in these cells appears to be more prolonged and less cytotoxic than in lymphocytes, and as a

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result, monocytes and macrophages represent important reservoirs of HIV infection. It has recently been discovered that macrophages may serve as reservoirs of HIV infection even in certain AIDS patients who test negative 5 for the presence of HIV antibodies. No effective cure is available for AIDS, although dideoxynucleosides have been shown to prolong life and to reduce the incidence of certain fatal infections associated with AIDS.

Certain monocyte-derived macrophages, when infected 10 with some strains of HIV, have been found to be resistant to treatment with dideoxycytidine, azidothymidine, and other dideoxynucleosides *in vitro* as shown by Richman, et al. (1). The resistance may be due in part to the low 15 levels of dideoxynucleoside kinase which result in a reduced ability to phosphorylate AZT, ddC or ddA. Clearly, it would be useful to have more effective ways of delivering large amounts of effective antiviral compounds to macrophages infected with HIV or other viruses and other 20 cells having viral infections. It would also be useful to have more effective ways of delivering antiviral compounds which not only increase their potency but prolong their 25 efficacy.

Dideoxynucleoside analogues such as AZT are the most potent agents currently known for treating AIDS, but in a 25 recent human trial, serious toxicity was noted, evidenced by anemia (24%) and granulocytopenia (16%) (2,3). It is desirable, therefore, to provide a means for administering AZT and other dideoxynucleosides in a manner such that the toxic side effects of these drugs are reduced. Further, 30 it is desirable to provide selective targeting of the dideoxynucleoside to monocyte/macrophages to enhance the efficiency of the drug against viral infection in this group of cells. One way to do this is to take advantage of the uptake of liposomes by macrophages.

35 In 1965, Alex Bangham and coworkers discovered that dried films of phosphatidylcholine spontaneously formed

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closed bimolecular leaflet vesicles upon hydration (4). Eventually, these structures came to be known as liposomes.

5 A number of uses for liposomes have been proposed in medicine. Some of these uses are as carriers to deliver therapeutic agents to target organs. The agents are encapsulated during the process of liposome formation and released *in vivo* when liposomes fuse with the lipids of cell surface membrane. Liposomes provide a means of 10 delivering higher concentrations of therapeutic agents to target organs. Further, since liposomal delivery focuses therapy at the site of liposome uptake, it reduces toxic side effects.

15 For example, liposomal antimonial drugs are several hundred-fold more effective than the free drug in treating leishmaniasis as shown independently by Black and Watson (5) and Alving, et al. (6). Liposome-entrapped amphotericin B appears to be more effective than the free drug in treating immunosuppressed patients with systemic 20 fungal disease (7). Other uses for liposome encapsulation include restriction of doxorubicin toxicity (8) and diminution of aminoglycoside toxicity (9).

25 As previously mentioned, it is now thought that macrophages are an important reservoir of HIV infection (10, 11). Macrophages are also a primary site of liposome uptake (12, 13). Accordingly, it would be desirable to utilize liposomes to enhance the effectiveness of antiviral nucleoside analogues in treating AIDS and other viral infections.

30 The use of liposomes to deliver phosphorylated dideoxynucleoside to AIDS infected cells which have become resistant to therapy has been proposed in order to bypass the low dideoxynucleoside kinase levels.

35 Attempts have also been made to incorporate nucleoside analogues, such as iododeoxyuridine (IUDR), acylovir (ACV) and ribavirin into liposomes for treating diseases other

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than AIDS. However, these attempts have not been entirely satisfactory because these relatively small water soluble nucleoside analogues tend to leak out of the liposome rapidly (14, 15), resulting in decreased targeting effectiveness. Other disadvantages include the tendency to leak out of liposomes in the presence of serum, difficulties in liposome formulation and stability, low degree of liposomal loading, and hydrolysis of liposomal dideoxynucleoside phosphates when exposed to acid hydrolases after cellular uptake of the liposomes.

Attempts have also been made to combine nucleoside analogues, such as arabinofuranosylcytosine (ara-C) and arabinofuranosyladenine (ara-A), with phospholipids in order to enhance their catabolic stability as chemotherapeutic agents in the treatment of various types of cancer (16). The resulting agents showed a decreased toxicity and increased stability over the unincorporated nucleoside analogues. However, the resulting agents exhibited poor cellular uptake (16) and poor drug absorption (17).

In order to use nucleoside analogues incorporated into liposomes for treating viral infections more effectively, it is desirable to increase the stability of the association between the liposome and the nucleoside analogue.

In order to further enhance the effectiveness of these antiviral liposomes, it would be desirable to target the liposomes to infected cells or sites of infection. Greater specificity in liposomal delivery may be obtained by incorporating monoclonal antibodies or other ligands into the liposomes. Such ligands will target the liposomes to sites of liposome uptake capable of binding the ligands. Two different approaches for incorporating antibodies into liposomes to create immunoliposomes have been described: that of Huang and coworkers (18) involving the synthesis of palmitoyl antibody, and that of Leserman, et al. (19)

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involving the linkage of thiolated antibody to liposome-incorporated phosphatidylethanolamine (PE).

The methods disclosed here apply not only to dideoxynucleosides used in the treatment of AIDS and other 5 retroviral diseases, but also to the use of antiviral nucleosides in the treatment of diseases caused by other viruses, such as herpes simplex virus (HSV), human herpes virus 6, cytomegalovirus (CMV), hepatitis B virus, Epstein-Barr virus (EBV), and varicella zoster virus (VZV). Thus, 10 the term "nucleoside analogues" is used herein to refer to compounds that can inhibit viral replication at various steps, including inhibition of viral reverse transcriptase or which can be incorporated into viral DNA or RNA, where they exhibit a chain-terminating function.

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Summary of the Invention

The invention provides compounds and compositions for use in treating viral infections, including HIV (AIDS), herpes simplex virus (HSV), human herpes virus 6, 20 cytomegalovirus (CMV), hepatitis B virus, Epstein-Barr virus (EBV), and varicella zoster virus (VZV). A composition may contain, in addition to a pharmaceutically acceptable carrier, a lipophilic antiviral compound prepared by chemically linking an antiviral nucleoside 25 analogue to at least one lipid species. The antiviral nucleoside analogue may be linked to the lipid through a monophosphate, diphosphate or triphosphate group. The invention, further, provides a method for incorporating such lipid derivatives of antiviral agents into liposomes 30 for improved delivery of the antiviral agent. A liposome comprises a relatively spherical bilayer which is comprised wholly or in part of the above-described lipid derivatives of antiviral agents. The liposome may also contain pharmacologically inactive lipids. Further, the liposome 35 may contain a ligand, such as a monoclonal antibody to a viral binding site (such as CD4), or other binding protein.

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Such a ligand provides additional specificity in the delivery site of the antiviral agent. The invention provides a method for incorporating such ligands into antiviral liposomes.

5 Thus, according to the invention there is provided a compound having antiviral properties, comprising:

10 a nucleoside analogue having a base portion comprising a purine or pyrimidine or analogue thereof, and a sugar portion comprising a pentose residue, wherein at least one said portion is a non-naturally occurring nucleoside component; and

15 a lipid moiety linked to said pentose residue; with the proviso that said compound is in the form of a liposome when said pentose residue is arabinofuranose and said base portion is cytosine or adenine.

20 In one preferred embodiment, the compound is a phosphatidylidideoxynucleoside or a dideoxynucleoside diphosphate diglyceride. In another, the lipid species may comprise at least one acyl ester, ether, or vinyl ether group of glycerol-phosphate. Phosphatidic acids having at least one acyl ester, ether, or vinyl ether group may also serve as a favored lipid species.

25 In another embodiment, the nucleoside analogue is a purine or pyrimidine linked through a β -N-glycosyl bond to a pentose residue that lacks at least one of the 2' or 3' carbons, but retains the 5' carbon, and the phosphate group is bound to the 5' carbon (i.e., what would have been the 5' carbon in a complete pentose moiety). In another 30 embodiment of the invention, the lipid species is an N-acyl sphingosine.

35 In some preferred embodiments, the acyl or alkyl groups of the lipid species, of whatever linkage, as for example ester, ether or vinyl ether, comprise 2 to 24 carbon atoms. In one variation, at least one of the acyl or alkyl groups is saturated. In another, at least one of

the acyl or alkyl groups has up to six double bonds. In yet another embodiment, an acyl or alkyl group may be attached directly by ester or alkyl linkage to the 5'-hydroxyl of the nucleoside.

5 In still another, the lipid moiety is a glyceride and the glyceride has two acyl groups that are the same or different. In still another embodiment of the invention, the lipid species is a fatty alcohol residue which is joined to a phosphate linking group through an ester bond.

10 The compound may advantageously have from one to three phosphate groups, and at least one fatty alcohol ester, and may have two or more fatty alcohol residues that are the same or different in structure. These fatty alcohols are preferably linked to the terminal phosphate group of

15 the compound.

Moreover, the invention includes a composition wherein, in addition to the compound, the liposome further comprises phospholipids selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, 20 phosphatidylglycerol, phosphatidylserine, phosphatidylinositol and sphingomyelin.

In one embodiment of the invention, the percentage of antiviral agent is 0.01 to 100 percent by weight of the liposome.

25 In another embodiment, the liposome further comprises a ligand bound to a lipid substrate. The ligand may be an antibody, such as a monoclonal antibody to a viral antigen. The viral antigen could be gp41 or gp110 of HIV, or could be any other suitable viral antigen. In one embodiment, 30 the ligand is CD4 receptor protein, or CD4 protein itself. Alternatively, the ligand is an antibody to CD4 or a protein or other substance that binds CD4.

The invention also contemplates a composition for use in treating viral and retroviral infections, comprising a 35 liposome formed at least in part of an lipophilic antiviral agent, the agent comprising a nucleoside analogue having a

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base and a pentose residue with at least one lipid species attached to the nucleoside analogue through a monophosphate, diphosphate or triphosphate linking group at the 5' hydroxyl of the pentose residue of the nucleoside analogue, and a pharmaceutically acceptable carrier therefore.

Thus, there is provided a compound having antiviral properties, comprising an antiviral nucleoside analogue having a base portion comprising a substituted or unsubstituted purine or pyrimidine, and a sugar portion comprising a pentose residue, and a lipid moiety linked to the pentose residue, with the proviso that the compound is in the form of a liposome when the pentose residue is ribose and the base portion is cytosine, and when the pentose residue is arabinofuranose and the base portion is cytosine or adenine. In one embodiment, the nucleoside analogue is a nitrogenous base which is a purine, pyrimidine, or a derivative thereof, and the pentose residue is a 2',3'-dideoxy, 2',3'-didehydro, azido or halo derivative of ribose, or an acyclic hydroxylated fragment of ribose. The pentose residue may thus be a 2',3'-dideoxyribose, and the nucleoside analogue may be 2',3'-dideoxycytidine, 2',3'-dideoxythymidine, 2',3'-dideoxyguanosine, 2',3'-dideoxyadenosine, 2',3'-dideoxyinosine, or 2,6 diaminopurine, 2',3'-dideoxyriboside.

In another embodiment, the pentose residue is a 2',3'-didehydroribose and the nucleoside is 2',3'-didehydrothymidine, 2',3'-didehydrocytidine carbocyclic, or 2',3'-didehydroguanosine.

In still another embodiment, the pentose residue is an azide derivative of ribose, and the nucleoside is 3'-azido-3'-deoxythymidine, 3'-azido-3'-deoxyguanosine, or 2,6-diaminopurine-3-azido-2',3'dideoxyriboside.

35 In still another embodiment of the invention, the pentose residue is a halo derivative of ribose and the

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nucleoside is 3'-fluoro-3'-deoxythymidine, 3'-fluoro-2',3'-dideoxyguanosine, 2',3'-dideoxy-2'-fluoro-ara-adenosine, or 2,6-diaminopurine-3'-fluoro-2',3'-dideoxyriboside. The 5 invention also includes halo derivatives of the purine or pyrimidine rings, such as, for example, 2-chloro-deoxyadenosine. Alternatively, the pentose residue is an acyclic hydroxylated fragment of ribose, and the nucleoside is 9-(4,-hydroxy-1',2'-butadienyl) adenine, 10 3-(4,-hydroxy-1',2'-butadienyl) cytosine, 9-(2-phosphonylmethoxyethyl)adenine or phosphonomethoxydiaminopurine.

In accordance with another aspect of the invention, the nucleoside analogue is acyclovir, gancyclovir, 1-(2'-deoxy-2'-fluoro-1- β -D-arabinofuranosyl)-5-iodocytosine (FIAC) or 1(2'-deoxy-2'-fluoro-1- β -D-arabinofuranosyl)-5-iodouracil (FIAU).

In all of the foregoing compounds, a monophosphate, diphosphate, or triphosphate linking group may be provided 20 between the 5' position of the pentose residue and the lipid species. Alternatively, there may be an aliphatic bridge comprising two functional groups and having from 0 to 10 carbon atoms between the functional groups, the bridge joining the lipid and the pentose residue. In still 25 further embodiments of the invention, the lipid species is a fatty acid, a monoacylglycerol, a diacylglycerol, or a phospholipid. The phospholipid may have a head group comprising a sugar or a polyhydric alcohol. Specific examples of phospholipids include bis(diacylglycero)- 30 phosphate and diphosphatidylglycerol. Other examples of lipid species include D,L-2,3-diacyloxypropyl-(dimethyl)-beta-hydroxyethyl ammonium groups.

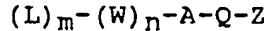
In accordance with another aspect of the present invention, the lipid species comprises from 1 to 4 fatty acid moieties, each the moiety comprising from 2 to 24 35 carbon atoms. Advantageously, at least one fatty acid

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moiety of the lipid species is unsaturated, and has from 1 to 6 double bonds.

Particular examples of these compounds include 3-phosphonomethoxyethyl-2,6-diaminopurine; 1,2-
5 diacylglycerophospho-5'-(2',3'-dideoxy)thymidine.

Specific compounds are provided having the formula:

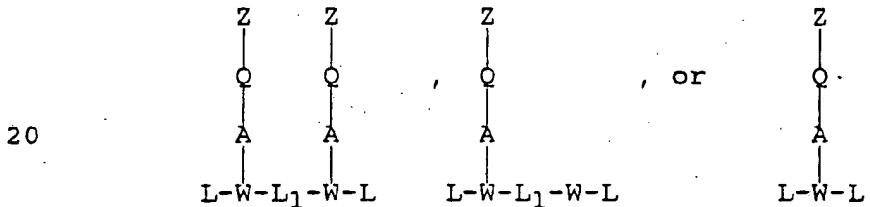


wherein

10 Z is the base portion of the nucleoside analogue, Q is the pentose residue, A is O, C, or S, W is phosphate, n = 0 to 3, and L is a lipid moiety wherein m = 1 to 5, and wherein each L is linked directly to a W except when n=0, in which case each L is linked directly to A.

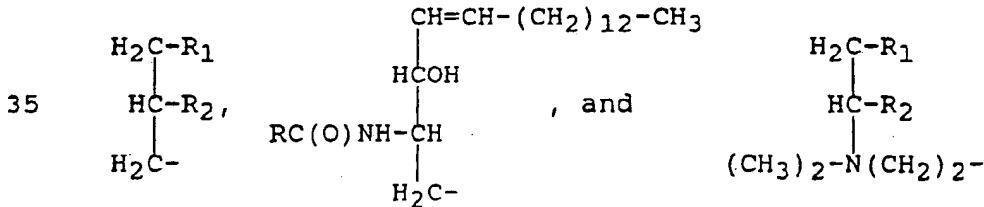
Also included are compounds having the formula:

15



20 wherein Z is the substituted or unsubstituted purine or pyrimidine group of the nucleoside analogue,
25 Q is the pentose residue,
W is phosphate, A is O, C, or S, L₁ is (CH₂-CHOH-CH₂), and L is a lipid moiety.

30 In one embodiment of the invention, with reference to the foregoing formulas, each L is independently selected from the group consisting of R,



40 wherein R, R₁ and R₂ are independently C₂ to C₂₄ aliphatic

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groups and wherein R, R₁ and R₂ independently have from 0 to 6 sites of unsaturation, and have the structure
 $\text{CH}_3-(\text{CH}_2)_a-(\text{CH}=\text{CH}-\text{CH}_2)_b-(\text{CH}_2)_c-\text{Y}$
wherein the sum of a and c is from 1 to 23, and b is 0 to 5, and wherein Y is C(O)O-, C-O-, C=C-O-, C(O)S-, C-S-, or C=C-S-.

In one embodiment of the foregoing compounds, the pentose residue comprises ribose, dideoxyribose, didehydroribose, or an azido or halosubstituted ribose, 10 attached at the 9 position of the purine or at the 1 position of the pyrimidine.

The present invention also provides a method for synthesizing a lipid derivative of an antiviral nucleoside, comprising the step of reacting an antiviral nucleoside, 15 having a ribose hydroxyl group, with a phospholipid in the presence of a coupling reagent whereby the nucleoside is joined to the phospholipid by a phosphate bond at the position of the ribose hydroxyl group. In one preferred embodiment, the phospholipid is a diacyl phosphate. In 20 another, the phospholipid is a phosphatidic acid or a ceramide. Also provided herein is a method of synthesizing a lipid derivative of an antiviral nucleoside, comprising the steps of reacting an antiviral nucleoside monophosphate with a reagent HL, wherein L represents a 25 leaving group, to form a nucleoside PO₄-L, reacting the nucleoside PO₄-L with a phosphatidic acid to bind the acid to the nucleoside through a pyrophosphate bond. In one variation of the method, the nucleoside monophosphate is AZT 5'-monophosphate.

Still a further method provided by the present invention is a method of synthesizing a glyceride derivative of a nucleoside analogue, comprising the step of joining a monoglyceride or diglyceride and an antiviral nucleoside monophosphate with a coupling agent in the 35 presence of a basic catalyst. In one embodiment, the

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glyceride is 1-O-stearoylglycerol and the nucleoside is AZT monophosphate.

Also a part of the present invention is a method for preparing a suspension of liposomes for use in treating 5 viral and retroviral infections in a mammal, comprising providing a lipophilic antiviral agent comprising at least one lipid species attached to a nucleoside analogue through a monophosphate, diphosphate or triphosphate linking group at the 5' position of the pentose residue of the 10 nucleoside, combining the lipophilic antiviral agent and a pharmacologically acceptable aqueous solvent to form a mixture, and forming liposomes from the lipophilic antiviral agent. The liposomes may be formed, for example, by sonication, extrusion or microfluidization. In one 15 preferred embodiment, the combining step further comprises including in the combination a pharmacologically inactive lipophilic lipid. This inactive lipid can be, for example, a phosphatidylethanolamine, a sphingolipid, a sterol or a glycerophosphatide. The method also may include treating 20 the liposomes with thio-antibodies to produce immunoliposomes, or including in the combination an lipophilic lipid which is, in part, comprised of a ligand. Thus, the liposome may include a ligand bound to a lipid substrate.

25 In addition, the invention includes a method for treating retroviral and viral infections in a mammal, such as a human, by administering a sufficient quantity of the antiviral nucleoside analogues described herein to deliver a therapeutic dose of the antiviral agent to the mammal. 30 In a preferred embodiment, the method is used to treat retroviral and viral infections in a mammal, wherein the retrovirus has become resistant to therapy with conventional forms of an antiviral agent. The present invention also includes a method for treatment of patients 35 having strains of HIV that have developed resistance to AZT or reduced sensitivity to AZT, comprising the step of

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administering a compound of the present invention to such patient in an effective, retrovirus-inhibiting dosage. Also included in the present invention is a method for treating a viral infection in a mammal,
5 comprising the step of administering an effective amount of a compound as described herein to a mammal. The infection may be a herpes simplex infection, and the compound may be phosphatidylacyclovir. Alternatively, the virus may be HIV retrovirus, and the compound may be 5'-palmitoylAZT.
10 The method includes use where the retrovirus is a strain of HIV that has developed resistance to a nucleoside analogue.

Also disclosed herein is a method for prolonging the antiviral effect of a nucleoside analogue in a mammal, comprising administering the nucleoside analogue to the
15 mammal in the form of the nucleoside-lipid derivatives disclosed herein. Also disclosed is a method for avoiding or overcoming resistance of the retrovirus to nucleoside analogues through administering the analogue in the form of the lipid derivative compounds disclosed herein.

20 The present invention includes use of the compounds and compositions of the invention in the preparation of a medicament for treatment of a human viral infection. The compositions of the invention may comprise a compound of the invention and a pharmaceutically acceptable carrier.
25 Compositions of the invention may comprise a compound of the invention and at least one other antiviral compound.

30 Liposomal delivery of antiretroviral and antiviral drugs results in higher dosing of macrophage and monocyte cells which take up liposomes readily. The unique advantages of the present invention are that the lipid derivatives of the antiviral nucleosides are incorporated predominantly into the phospholipid layer of the liposome rather than in the aqueous compartment. This allows larger quantities of antiviral analogue to be incorporated in
35 liposomes than is the case when water soluble phosphate esters of the nucleosides are used. Complete incorporation

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of the antiviral derivative into liposomes will be obtained, thus improving both the drug to lipid ratio and the efficiency of formulation. Further, there will be no leakage of the antiviral lipid analogues from the liposome 5 during storage. Finally, liposomal therapy using these compounds allows larger amounts of antiviral compound to be delivered to the infected macrophage and monocyte cells. Therapy with liposomal compounds containing site specific ligands allows still greater amounts of antiviral 10 compounds to be delivered with increased specificity.

Another novel advantage of this invention is that each class of lipid derivatives of antiviral nucleosides disclosed below is believed to give rise directly to antiviral phosphorylated or non-phosphorylated nucleosides 15 upon cellular metabolism.

A further advantage of this invention is that the novel lipid derivatives are incorporated into the cell, protecting the cell for prolonged periods of time, up to or exceeding 48 hours after the drug is removed.

20 These and other advantages and features of the present invention will become more fully apparent from the following description and appended claims.

Brief Description of the Drawings

25 Figures 1-5 are graphs plotting p24 production by HIV-infected cells as a function of the amount of the compound of the present invention administered *in vitro*.

Detailed Description of the Invention

30 The present invention involves lipid derivatives of nucleoside analogues which can be incorporated into the lipid bilayer of liposomes. These derivatives are converted into nucleoside analogues by constituent cellular metabolic processes, and have antiviral effects *in* 35 *vivo* and *in vitro*.

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Suitable lipid derivatives of nucleoside analogues comprise phosphatidyl nucleosides, nucleoside diphosphate diacylglycerols, nucleoside acyl phosphates, and ceramide phosphonucleosides. With the exception of the acyl phosphates, which can include from one to five acyl groups, the lipid derivatives of these compounds provide one or two hydrophobic acyl groups to anchor the nucleoside in the lipid bilayer of the liposome. The present invention also comprises lipid derivatives capable of providing additional acyl groups, and hence greater anchoring strength for nucleoside analogues. The increase in anchoring strength makes it possible to utilize nucleoside analogues of greater polarity in liposome formulations. Accordingly we disclose additional nucleoside structures of this type for use in liposomal therapies. We also disclose lipid derivatives of nucleoside analogues in which the lipid group is directly attached to the nucleoside, rather than through a phosphate link.

20

Nomenclature:

The lipid derivatives of the present invention are made up of complex structures which can only be rigorously defined by cumbersome terminology. For purposes of clarity, the descriptions of lipid and nucleosides components and their combinations will be in terms of commonly used trivial names, familiar to those in the art. For example, the well known drug, 3'-azido-3'-deoxythymidine, will be frequently referred to as AZT. Similarly the derivative of AZT comprising a 1,2 diacylglycerol-3-phosphate moiety, will be frequently referred to as phosphatidylAZT or pAZT. Parallel derivatives of dideoxythymidine or dideoxycytidine will correspondingly be referred to as phosphatidylddT or pddT and phosphatidylddC and pddC. Derivatives of halogenated

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nucleosides will be referred to as, for example, phosphatidyl-3'-BrdT.

The nucleoside analogues of the invention can be any nucleoside that does not occur naturally in the species to be treated for viral infection. It may comprise a naturally occurring purine or pyrimidine base attached to an analogue of a naturally occurring ribose group. It may likewise comprise an analogue of a purine or pyrimidine base attached to a ribose or deoxyribose group which is present in naturally occurring nucleosides. Alternatively, both the base and the ribose moieties of the nucleoside analogues may be analogues of those found in nature. A nucleoside analogue may also comprise either a normal base or a base analogue attached to a non-ribose sugar moiety.

Analogues of both the purine or pyrimidine base and the ribose group can differ from a corresponding naturally occurring moiety by having new substituent groups attached thereto, by having naturally occurring substituent groups deleted therefrom, or by having atoms normally present replaced by others. Examples of analogues formed by substitution are 2,6-diaminopurine and 3'-azido-3'-deoxyribose; by deletion, 6-oxypurine or didehydroribose; by replacement, 8-azaguanine.

Nucleoside analogues may also comprise a purine or pyrimidine base attached to the pentose moiety in a non-naturally occurring linkage, such as, for example through the nitrogen at the 3 position rather than the 1 position of the pyrimidines.

In general, the nucleoside analogues used in preparing the liposomes of the present invention will have a purine or pyrimidine base, e.g., adenine, guanine, cytosine or thymine, or an analogue thereof, attached to a pentose, such as ribose or a ribose residue and/or derivative. The attachment is through the nitrogen in the 9 position of the purines and through the nitrogen in the 1

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position of the pyrimidines. These nitrogens are linked by a β -N-glycosyl linkage to carbon 1 of the pentose residue.

5 The pentose residue may be a complete pentose, or a derivative such as a deoxy- or dideoxypentose. In addition, the pentose residue can be a fragment of a pentose, such as a hydroxylated 2-propoxymethyl residue or a hydroxylated ethoxymethyl residue. Particular nucleoside residues having these structures include acyclovir and 10 gancyclovir. The pentose may also have an oxygen or sulfur substitution for a carbon atom at, for example, the 3'position of deoxyribose (BCH-189).

15 The phosphate groups are generally connected to the 5' carbon of the pentoses in the compounds of the present invention; however, compounds wherein the phosphate groups are attached to the 3' hydroxyl group of the pentose are within the invention if they possess antiviral activity. Where lipids are linked directly to pentose groups, those linkages may also be made either through the 3' or 20 preferably through the 5' pentose carbon.

25 It is important to recognize that in compounds having pentose residues that are not complete pentoses, the phosphate groups are connected to the carbon that would have been the 5' carbon if the pentose were complete. In these pentose fragments, the 2' and/or 3' carbons may be missing; nevertheless, they are considered to be nucleoside derivatives within the meaning of present invention, and the carbon atom to which the phosphate groups are connected will generally be referred to herein as the 5' carbon for 30 purposes of consistency of usage.

35 Any lipid derivative of a nucleoside analogue having an antiviral activity is within the scope of the invention. The antiviral activity may reside in any component of the lipid-nucleoside complex, that is, in a nucleoside base analogue, in a ribose analogue, or in the substitution of another pentose for ribose. It may also reside in the

-19-

complex as a whole, wherein, for example, a weakly antiviral analogue or one possessing imperceptible or latent viral activity becomes more potent following its incorporation into a lipid derivative of a nucleotide.

5 Nucleosides known to have such activity are members of the class comprising 3'-azido-2',3'-dideoxypyrimidine nucleosides, for example, AZT, AZT-P-AZT, AZT-P-ddA, AZT-P-ddI, AzddClU, AzddMeC, AzddMeC N4-OH, AzddMeC N4Me, AZT-P-CyE-ddA, AzddEtU(CS-85), AzddU(CS-87), AzddC(CS-91),
10 AzddFC, AzddBrU, and AzddIU; the class comprising 3'-halopyrimidine dideoxynucleosides, for example, 3-FddClU, 3-FddU, 3-FddT, 3-FddBrU, and 3-FddEtU; the class comprising 2',3'-didehydro-2',3'-dideoxynucleosides (D4 nucleosides), for example, D4T, D4C, D4MeC, and D4A; the
15 class comprising 2',3'-unsubstituted dideoxypyrimidine nucleosides, for example, 5-F-ddC, ddC and ddT; the class comprising 2',3'-unsubstituted dideoxypurines nucleosides, for example, ddA, ddDAPR(diaminopurine), ddG, ddI, and ddMeA(N6 methyl); and the class comprising sugar-
20 substituted dideoxypurine nucleosides, for example, 3-N₃ddDAPR, 3-N₃ddG, 3-FddDAPR, 3-FddG, 3-FddaraA, and 3-FddA, wherein Me is methyl, Et is ethyl and CyEt is cyanoethyl.

25 Other suitable nucleotide analogues may be antiviral agents like acyclovir or gancyclovir (DHPG), or other analogues, as described below. Preferred dideoxy derivatives are those used in the treatment of AIDS, including 3'-azido-3'-deoxythymidine (azidothymidine or AZT); 2',3'-dideoxythymidine (ddT); 2',3'-dideoxycytidine
30 (ddC); 2',3'-dideoxyadenosine (ddA); and 2',3'-dideoxyguanosine (ddG). AZT, ddT, and ddC are most preferred analogues at present. The didehydropyrimidines, as well as carbovir, a carbocyclic 2',3'-didehydroguanosine, are also preferred. The 3'-azido derivatives of deoxyguanosine (AZG) and the pyrimidine, deoxyuridine, and the 3'-fluoro derivatives of

-20-

deoxythymidine and deoxyguanosine are preferred as well. Among the 2',6'-diaminopurines, the 2',3'-deoxyriboside and its 3'-fluoro and 3'-azido derivatives are preferred. Also preferred is 2-chloro-deoxyadenosine.

5 Among the acyclic sugar derivatives, 9-(4,-hydroxy-1',2'-butadienyl)adenine (adenallene) and its cytosine equivalent are preferred. Preferred acyclic derivatives having a purine or diaminopurine base are 9-(2-phosphonylmethoxyethyl)adenine and phosphonomethoxyethyl 10 deoxydiaminopurine (PMEDADP).

15 Stereoisomers of these nucleosides, such as 2'-fluoro-ara-ddA, may be advantageous because of their resistance to acid-catalyzed hydrolysis of the glycosidic bond, which prolongs their antiviral activity. In such cases, they are preferred.

20 For treating herpes, cytomegalovirus and hepatitis B infections, one may utilize the lipid derivatives of acyclovir, gancyclovir, 1-(2'-deoxy-2'-fluoro-1- β -D-arabinofuranosyl)-5-iodocytosine (FIAC) or 1(2'-deoxy-2'-fluoro-1- β -D-arabinofuranosyl)-5-iodouracil (FIAU).

25 The lipids are preferably attached to the nucleoside analogues through phosphate linkages. Lipid derivatives comprising a phosphate link between a nucleoside analogue and lipid may be prepared from phospholipids, phosphorylated nucleoside analogs, or both. Suitable phospholipids comprise phosphoglycerides, sphingolipids, or acyl phosphates.

30 Lipid derivatives of nucleoside analogue in which lipids are linked either through mono-, di-, or triphosphate groups may be prepared from phosphorylated nucleoside analogues. Phosphorylated nucleoside analogues are known. The dideoxynucleoside analogue is phosphorylated according to conventional procedures such as the phosphorous oxychloride method of Toorchen and Topal 35 (20). The preferred modified analogue is the 5'-monophosphate. Since AZT, ddC and other dideoxynucleosides

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have only the 5'-hydroxyl, only the 5'-monophosphate is formed during phosphorylation; however, in other analogues in which the 3'hydroxyl is present, a 3'-monophosphate can be formed. The diphosphate and triphosphate analogues of 5 antiviral nucleosides may also be used.

The aliphatic groups of the lipid moieties preferably have chain lengths of two to twenty-four carbon atoms and have zero to six double bonds. The aliphatic groups may be attached to the glycerol moiety by acyl, ether or vinyl 10 ether bonds.

Synthetic Methods:

The lipid-nucleotide compounds of the present invention can be synthesized according to general methods 15 applicable to all lipids and all antiviral nucleosides described below, as indicated in the flow diagram of Figure and demonstrated specifically in Examples 1 through 7.

Lipids comprising fatty acids, alcohols, glycerides and phospholipids may be purchased from commercial 20 suppliers (Avanti Polar Lipids, Inc., Pelham, Alabama 35124) or may be synthesized according to known methods. Antiviral nucleoside analogues are available from Aldrich, Milwaukee, Wisconsin or from Sigma, St. Louis, Missouri.

It is important that all traces of water be removed 25 from the reactants in order for the coupling reactions to proceed. Therefore, the lipids are first either freeze-dried by solvent evaporation under vacuum, or in a vacuum oven over P₂O₅. The reactions are also carried out under an inert gas, such as, for example, argon.

30 The compounds of the invention can be formed according to synthetic procedures which couple a phospholipid to a nucleoside analogue or which couple a phospholipid to a nucleoside analogue monophosphate or diphosphate, wherein the phosphate group is located on the ribose group of the 35 nucleoside, at either the 3' or preferably the 5' location.

Lipids suitable for coupling to nucleosides,

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comprising primarily long chain fatty acids or alcohols, monoglycerides or diglycerides, ceramides and other lipid species described below, may be phosphorylated by treatment with appropriate agents, for example using phenyl 5 phosphorodichloridate according to the procedure of Brown (32), by treatment with phosphorus oxychloride as in Example 6, or by other known phosphorylation procedures.

In the first type of synthesis, a phospholipid, such as, for example, a phosphatidic acid, is coupled to a 10 selected nucleoside analogue at either the 3' or 5' hydroxyl by means of a coupling agent, such as, for example, 2, 4, 6-triisopropylbenzenesulfonyl chloride in the presence of a basic catalyst, for example, anhydrous pyridine, at room temperature. Other coupling agents, such 15 as dicyclohexylcarbodiimide can be used.

Lipid derivatives may also be synthesized by coupling a phosphatidic acid to an antiviral nucleoside monophosphate through a pyrophosphate bond. In this procedure, the nucleoside monophosphate or diphosphate is 20 converted to a derivative having a leaving group, for example, morpholine, attached to the terminal phosphate group, according to the procedure of Agranoff and Suomi (21) and as illustrated in Example 4, for preparing a derivative of AZT and Example 6, for a derivative of ddA. 25 A coupling of the phosphatidic acid and the nucleoside phosphate morpholidate occurs on treatment of a dry mixture of the two reactants with a basic catalyst, such as anhydrous pyridine, at room temperature.

The reactions are followed using thin layer 30 chromatography (TLC) and appropriate solvents. When the reaction, as determined by TLC is complete, the product is extracted with an organic solvent and purified by chromatography on a support suitable for lipid separation, for example, silicic acid.

35 The synthesis of products comprising adenine or cytidine having reactive amino groups may be facilitated by

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blocking those groups with acetate before the coupling reaction by treatment with acetic anhydride; after the chromatography of the final product, the amino groups are unblocked using ammonium hydroxide (Example 3).

5

Lipid Derivatives:

Compounds which will be most effective will have a lipid portion sufficient to be able to incorporate the material in a stable way into a liposomal bilayer or other 10 macromolecular array.

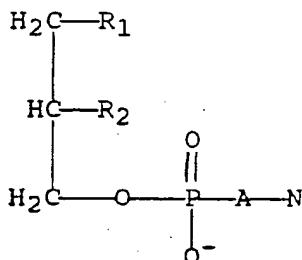
Some preferred lipid derivatives of nucleoside analogues that are within the scope of the present invention fall into four general classes:

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1. Antiviral phosphatidylnucleosides:

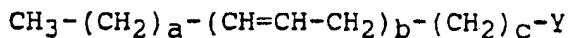
The structure of these antiviral lipid compounds is shown below:

20



25

where N is a "chain terminating" dideoxynucleoside such as AZT, ddc, ddA, dDI, or another antiviral nucleoside such as acyclovir or gancyclovir, A is a chalcogen (O, S or Se), and R₁ and R₂, which may be the same or different, are C₁ to C₂₄ aliphatic groups, having from 0 to 6 sites of 30 unsaturation, and preferably having the structure



wherein the sum of a and c is from 1 to 23; and b is 0 to 6; and wherein Y is C(O)O⁻, C-O⁻, C=C-O⁻, C(O)S-, C-S-, C=C-S-, forming acyl ester, ether or vinyl ether bonds,

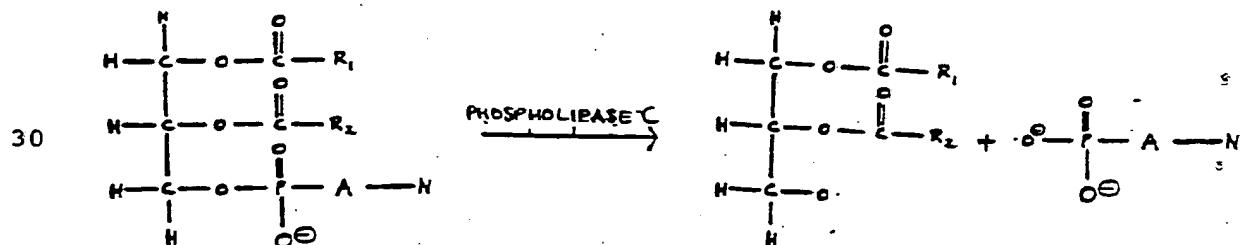
-24-

respectively, between the aliphatic groups and the glycerol moiety. These aliphatic groups in acyl ester linkage therefore comprise naturally occurring saturated fatty acids, such as lauric, myristic, palmitic, stearic, 5 arachidic and lignoceric, and the naturally occurring unsaturated fatty acids palmitoleic, oleic, linoleic, linolenic and arachidonic. Preferred embodiments comprise a monoester or diester, or a 1-ether, 2-acyl ester phosphatidyl derivative. In other embodiments, the 10 aliphatic groups can be branched chains of the same carbon number, and comprise primary or secondary alkanol or alkoxy groups, cyclopropane groups, and internal ether linkages.

This class of compounds may be prepared, for example, from the reaction of a diacylphosphatidic acid and an 15 antiviral nucleoside analogue in pyridine as described for the preparation of 1,2 dimyristoylglycerophospho-5'-(3'-azido-3'-deoxy)thymidine in Example 1.

Upon liposomal uptake, the compounds are believed to undergo metabolism by the phospholipases present in the 20 cell. For example, in the specific case of a diacylphosphatidyl derivative of a nucleoside, phospholipase C would act to give a diacylglycerol and the nucleoside monophosphate as shown below:

25

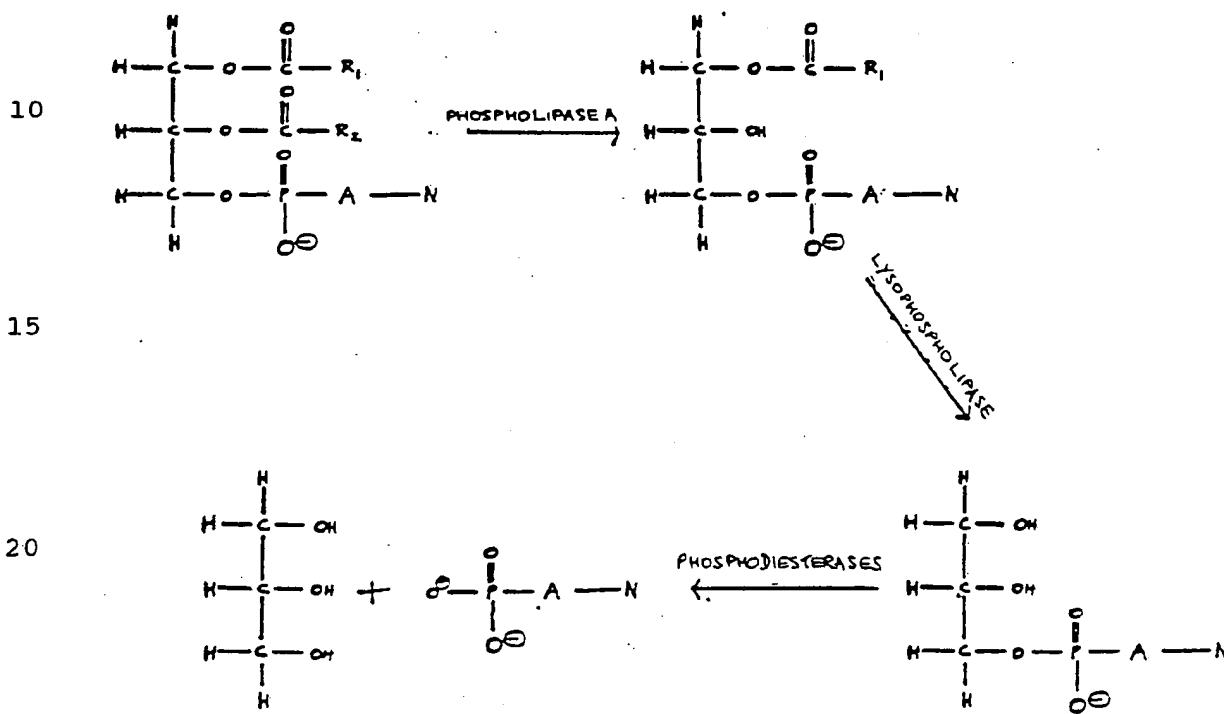


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-25-

Alternatively, the same phosphatidylnucleoside may be hydrolyzed by phospholipase A and lysophospholipase followed by phosphodiesterase to give glycerol and nucleoside monophosphate by the sequence shown below:

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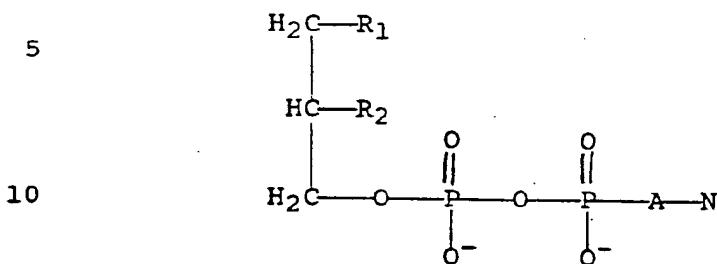
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2. Antiviral nucleoside diphosphate diglycerides:

The chemical structure of this class of compounds is shown below:



15 where N, A and R₁ and R₂ are as described above.

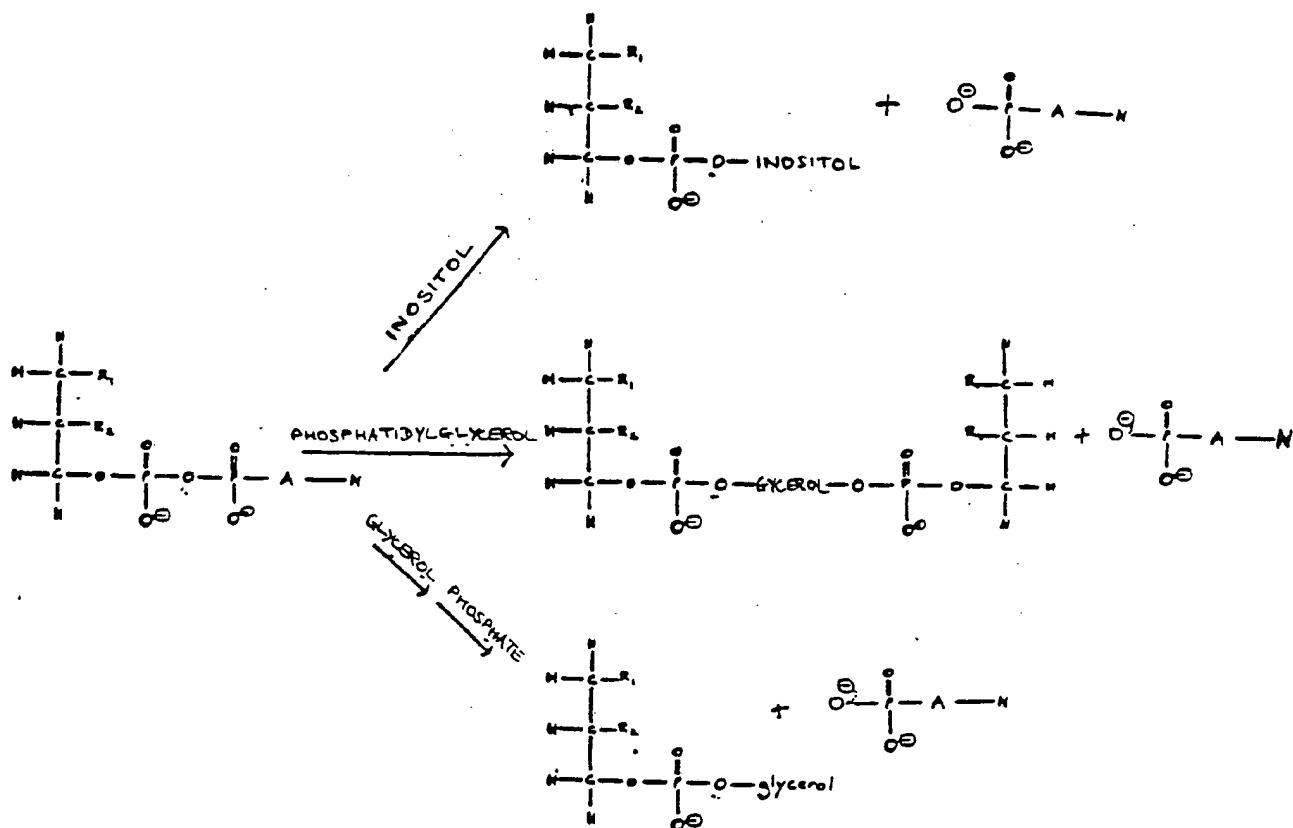
Nucleoside diphosphate diglycerides are known. The antiviral nucleoside diphosphate diglycerides may be prepared from phosphatidic acid and the antiviral nucleotide monophosphomorpholides by the method of 20 Agranoff and Suomi (21) as modified by Prottey and Hawthorne (22). This type of synthesis is presented in Example 4 for the synthesis of AZT 5'-diphosphate dipalmitoyl glycerol.

Upon liposomal delivery to cells, this class of 25 compounds will take part in several types of reactions since it is an analogue of CDP-diglyceride, an important naturally-occurring intermediate in the biosynthesis of phosphatidylglycerol, cardiolipin and phosphatidylinositol as shown below:

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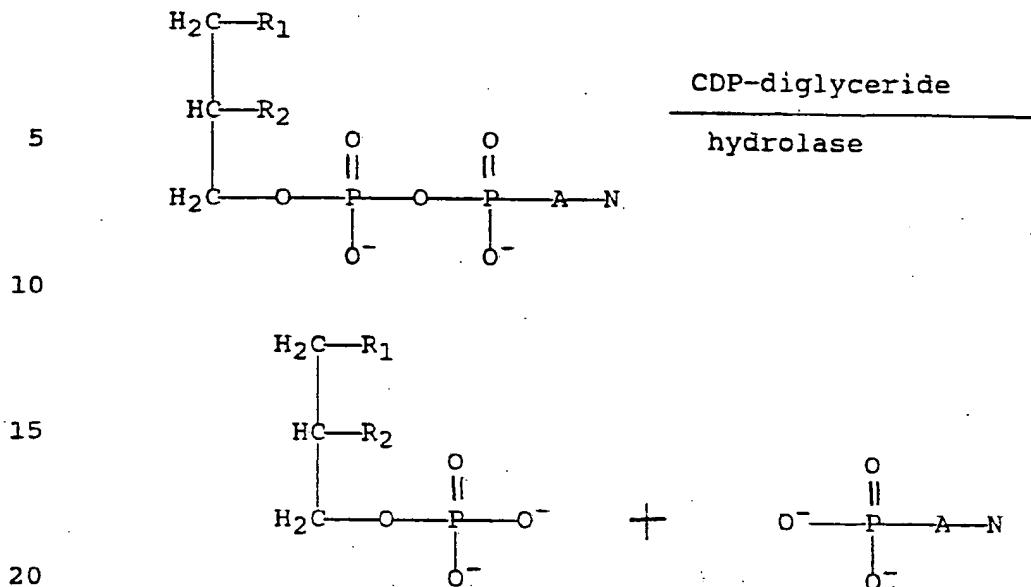
-27-



All of these reactions generate nucleoside monophosphate and a new phospholipid. It is important to note that Poorthuis and Hostetler (23) showed previously that a variety of nucleosides could substitute for CDP-diglyceride in these reactions, including UDP-diglyceride, 25 ADP-diglyceride and GDP-diglyceride (23). Significantly, Ter Scheggett, et al. (24) synthesized deoxy CDP-diglyceride and found that it could also replace CDP-diglyceride in the mitochondrial synthesis of phosphatidylglycerol and cardiolipin, thereby suggesting the possibility of using these novel compounds to generate 30 the antiviral nucleoside phosphates in the target cells.

CDP-diglyceride hydrolase catalyzes another important 35 metabolic conversion which gives rise to nucleoside monophosphate and phosphatidic acid, as shown below:

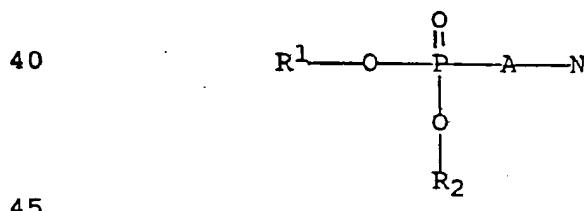
-28-



This pathway was first described in mammalian tissues by Rittenhouse, et al. (25). This enzyme, which is a pyrophosphatase, is expected to cleave dideoxynucleoside diphosphate diglyceride to the nucleoside monophosphate and phosphatidic acid, providing a second manner in which the nucleoside monophosphate can be formed in the target cells.

3. Antiviral nucleoside acyl phosphates:
 30 Another way to introduce a lipid compound into cells by means of liposomes is to synthesize acyl esters of the nucleoside monophosphates, diphosphates or triphosphates. This synthesis may be carried out according to the procedure in Example 5 for the synthesis of dihexadecyl phospho-5'-dideoxycytidine.

35 The structure of a diacylphosphonucleoside is shown below:



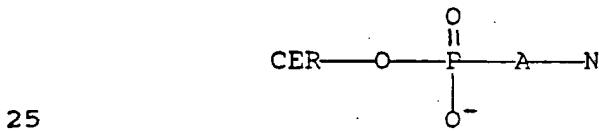
-29-

wherein N, A, R₁ and R₂ are as previously defined. In principle, one or more acid moieties of the phosphate may be esterified and many other combinations of phosphate and fatty alcohol substitution are possible. For example, a nucleoside monophosphate could have one or two aliphatic esters; a nucleoside diphosphate could have one to three aliphatic esters, and the nucleoside triphosphate could have one to four aliphatic esters. Nucleosides can be "chain terminating" dideoxynucleosides or other antiviral nucleosides.

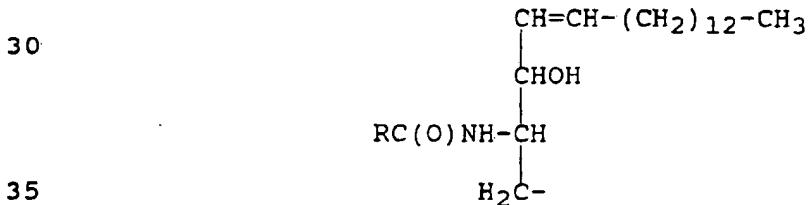
Since cells contain a variety of esterases, it is anticipated that this class of compounds will be hydrolyzed to the phosphorylated nucleoside, bypassing the deficiency of dideoxynucleoside kinase in human monocytes and macrophages, and thereby restoring the antiviral activity.

4. Ceramide antiviral phosphonucleosides:

Antiviral nucleoside phosphates can also be generated in cells after liposomal delivery of ceramide antiviral nucleoside phosphates having the general structure shown below:



where CER is an N-acylsphingosine having the structure:



wherein R is as defined previously, or an equivalent lipid-substituted derivative of sphingosine, and N is a "chain terminating" antiretroviral nucleoside or antiviral nucleoside as previously defined. This class of compounds is useful in liposomal formulation and therapy of AIDS and

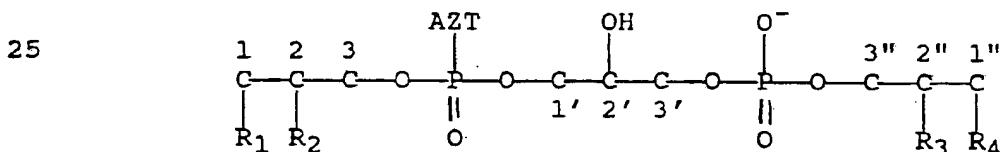
-30-

other viral diseases because it can be acted upon by sphingomyelinase or phosphodiesterases in cells giving rise to nucleoside monophosphate. In addition to the compound shown above, ceramide diphosphate dideoxynucleosides can also be synthesized, which may be degraded by cellular pyrophosphatases to give nucleoside monophosphate and ceramide phosphate.

5 Ceramide antiviral nucleoside phosphates may be prepared in a method similar to the method for preparing 10 antiviral nucleoside diphosphate diglycerides, with appropriate changes to the starting materials.

5. Other Lipid Derivatives of Antiviral Nucleosides

One approach to achieving even greater stability of 15 lipid derivatives of nucleoside analogues within liposomes is by increasing lipid-lipid interaction between the lipid-nucleoside structure and the bilayer. Accordingly, in preferred embodiments, lipid derivatives of nucleoside analogues having up to four lipophilic groups may be synthesized. One class of these comprises 20 diphosphatidylglycerol derivatives, having the general structure:



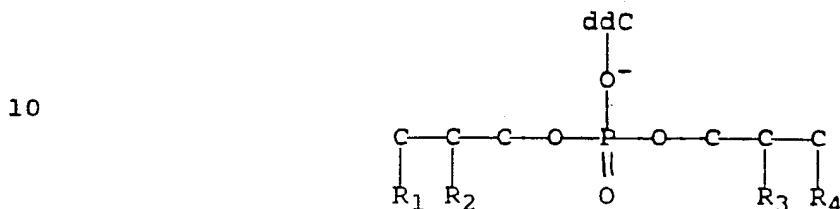
30

In this class, nucleosides are attached to one or both phosphates by a phosphodiester bond to the 5'-OH of the deoxyribose, ribose or dideoxyribose moiety of the 35 antiviral nucleoside. In the case of acyclic nucleosides, such as acyclovir or gancyclovir, the link would be to the OH group equivalent to that of the ribose, deoxyribose or dideoxyribose 5'-position. There may be one or two nucleosides attached to each molecule. Nucleoside

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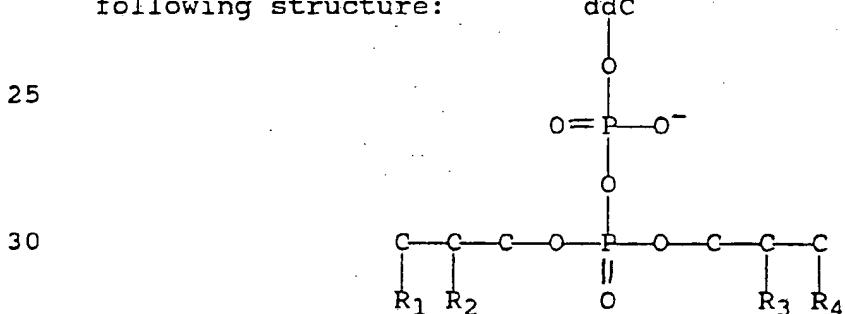
phosphates may also be attached by a pyrophosphate bond, as in Example 4.

Another class of derivatives having increased lipid components comprises bis(diacylglycero)phosphonucleotides, 5 having the general structure:



20 R_{1-4} may be two, three or four aliphatic groups which are independently R as previously defined, said groups being in acyl ester, ether, or vinyl ether linkages. This compound may be made by the method of Example 3.

The diphosphate version of this compound, with the following structure:



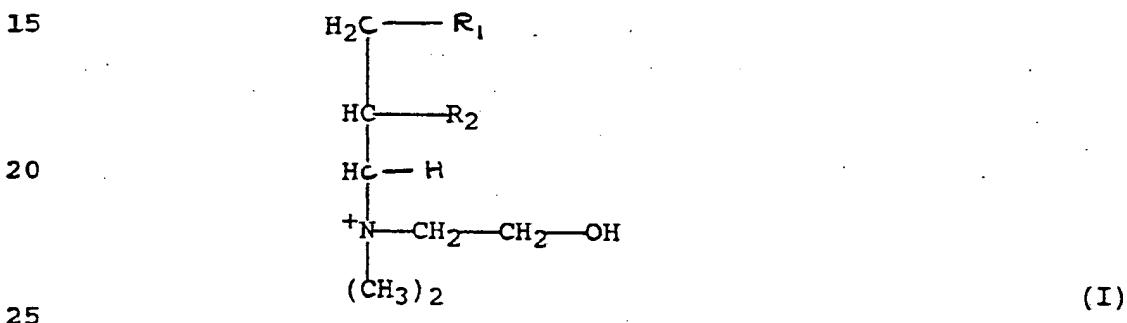
35 may be made by coupling the nucleoside monophosphomorpholidate to the phosphoester residue of bis(diacylglycero)phosphate according to the procedure of Example 4. This compound will be metabolized to AZT-P in the cells by CDP-diglyceride hydrolase (a pyrophosphatase). 40 These two types of compounds may provide superior metabolic and physical properties.

45 Other suitable lipid derivatives of nucleosides may be synthesized using novel lipids. It is desirable, for

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example, to synthesize phospholipid derivatives of antiviral and antiretroviral nucleosides which will give rise to potent antiviral agents upon alternate paths of metabolism by the target cells which take up the lipid 5 formulation. For derivatives made up of the following types of compounds, one might anticipate a cellular metabolism distinct from that of more conventional phospholipid derivatives, because these have a phosphate group which is removed from the usual lipid group by a 10 nitrogen containing group. The structure of these lipids features a quaternary ammonium derivative.

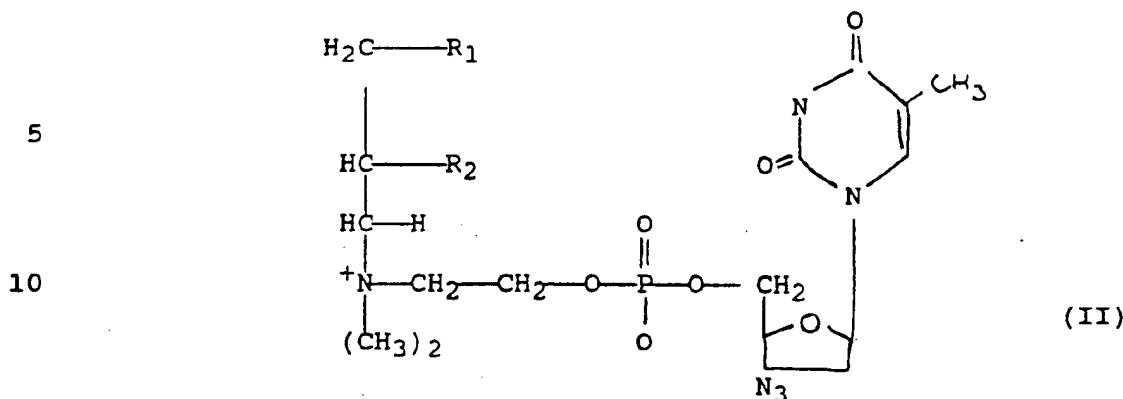
The compound shown:



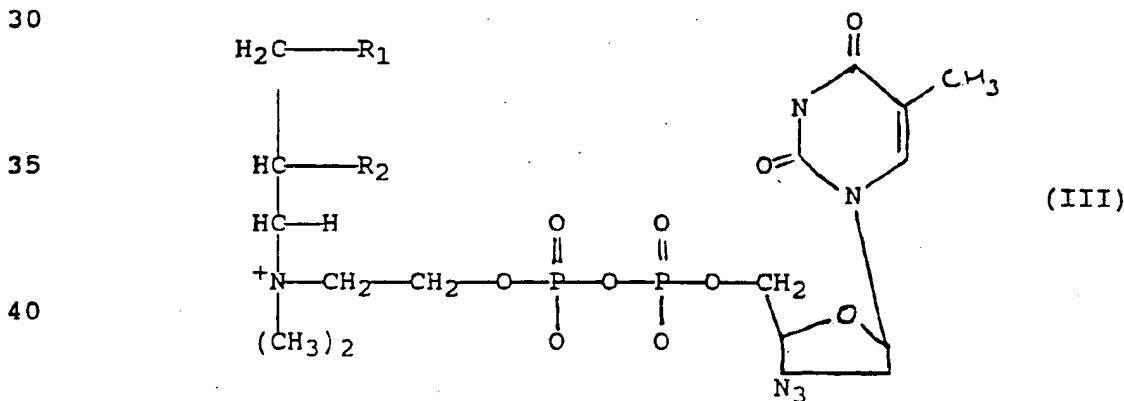
D,L,-2,3-distearoyloxypropyl(dimethyl)- β -hydroxyethyl ammonium acetate, was first synthesized by Rosenthal and Geyer in 1960 (35) and is available from Calbiochem, La 30 Jolla, California 92039. It can readily serve to link AZT-phosphate or any other antiviral nucleoside phosphate, using triisopropylbenzenesulfonyl chloride (TIBSC) as described in Example 1 or 7.

Alternatively, AZT may be linked to the phosphorylated 35 ammonium lipid prepared by POCl_3 , using TIBSC. Shown below is the AZT derivative of the phosphorylated compound I, D,L,-2,3-diacyloxypropyl(dimethyl)- β -hydroxyethyl ammonium acetate, where R_1 and R_2 are aliphatic groups as previously defined, of the preferred structure:

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Further, the Compound I of Rosenthal and Geyer may
 15 also be phosphorylated as they describe in their paper
 (35). One may also use the phosphorous oxychloride method
 of Toorchen and Topal (20) to prepare the phosphate ester
 of I. To this phosphorylated species one may then couple
 20 any antiviral or antiretroviral nucleoside using the
 morpholidate derivative of the nucleoside phosphate as
 reported by Agranoff and Suomi, (21) and modified by
 Prottey and Hawthorne, 1967 (22). The resulting
 nucleoside diphosphate derivatives of I may have exemplary
 25 properties as antiviral agents delivered in liposomes to
 infected cells. Preferred nucleosides include, but are
 not limited to: AZT, ddA, ddC, ddI, acyclovir, and
 gancyclovir. The AZT diphosphate derivative of Compound I
 is shown below:



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In any of the lipids derivatives described in the preceding sections 1 through 5 above, the nucleoside may be any antiviral nucleoside; R₁₋₂ (as well as R₃₋₄ for the bis(diacylglycero) species) may be any saturated or 5 unsaturated fatty acid having from 2 to 24 carbon atoms. Polyunsaturated, hydroxy, branched chain and cyclopropane fatty acids are also possible. The stereochemistry of the glycerol moieties can include sn-1 or sn-3 phosphoester bonds or racemic mixtures thereof. There may be 1 or 2, 10 (as well as 3, or 4 for the bis(diacylglycero) species) acyl ester groups, or alkyl ether or vinyl ether groups, as required.

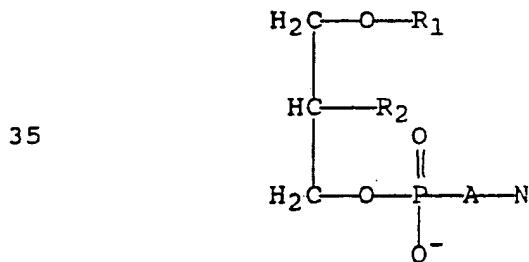
A variety of other phospholipids may be linked to nucleosides, including, but not limited to 15 phosphatidylglycerol, phosphatidylinositol, or any other phospholipid wherein the head group contains an available linking hydroxyl group, in either a natural polyhydroxyl alcohol such as inositol, or one in which it has been substituted by another polyhydroxy alcohol or by a 20 carbohydrate, such as a sugar, again either natural or synthetic. In this case the nucleoside phosphate will be added by esterification to one or more of the hydroxyls of the alcohol or carbohydrate. Other glycolipids may also serve as the ligand to which the phosphate group of the 25 nucleotide is attached by means of esterification to a glycolipid hydroxyl group. Other glycolipids, whether or not phospholipids, such as selected cerebrosides or gangliosides, either natural or synthetic, having suitable hydrophobic properties may also be advantageously used. 30 These may also be linked to nucleotides by similar esterification of carbohydrate hydroxyl groups.

Furthermore, antiviral nucleosides may be linked to 35 the phosphate groups of the phosphatidylinositol mono-, di- and triphosphates, or to the phosphate-substituted carbohydrate moieties of phospholipids or glycolipids, either natural or synthetic.

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Phosphatidylserine may be linked to nucleoside analogues directly by esterification of its carboxyl group with the 5'-hydroxyl of the nucleoside ribose group. Synthetic phospholipids which are similar in structure to phosphatidylserine, containing a carboxyl group in the polar headgroup, may be linked in a similar way.

Phospholipids having alkyl chains attached by ether or vinyl ether bonds may also be used to prepare nucleotide derivatives according to the present invention. Suitable phospholipids for this purpose comprise naturally occurring acetal phosphatides, or plasmalogens, comprising a long chain fatty acid group present in an unsaturated vinyl ether linkage. Alternatively, analogs of 1-O-alkyl glycerol or 2-O-alkyl glycerol may be prepared synthetically, and linked to a selected nucleotide as described in Example 7. Derivatives of glycero-3-phospho-5'-azidothymidine are preferred, and may be prepared by condensing AZT monophosphate with various analogs of 1-O-alkyl-glycerol having an alkyl group of 2 to 24 carbon atoms. The 1-O-alkyl group may consist of a saturated, unsaturated aliphatic group having a chain length of 2 to 24 carbon atoms. The 1-O-alkyl glycerol residue may be racemic or stereospecific. This compound may be acylated with fatty acid chlorides or anhydrides resulting in the synthesis of 1-O-alkyl, 2-acyl-glycero-3-phospho-5'azidothymidine. Similarly, by using a large excess of azidothymidine monophosphate, the 1-O-alkyl, 2,3-bis(phospho-5'-3'-azido, 3'-deoxythymidine)glycerol analogs may be synthesized. These derivatives have the general structure:



Where R_1 is an unsaturated or saturated alkyl chain 1 to 23 carbon atoms in length in ether or vinyl ether linkage. R_2 is OH or a saturated or unsaturated fatty acid ester of 5 2 to 24 carbon atoms. An ether or vinyl ether link at R_2 is also possible. The group at position 1 of glycerol may also be OH if R_2 is the ether linked alkyl chain. N is any antiviral nucleoside linked in a 5' phosphodiester link and A is a chalcogen (O, C or S).

10 Although phosphorylated antiviral nucleosides (nucleotides) are preferred embodiments of the present invention, it is possible to utilize non-phosphorus containing lipid derivatives of nucleoside analogues if it is not necessary to provide the infected cell with the 15 nucleoside phosphate in order to achieve an antiviral effect through the processes of cellular metabolism. Some examples of compounds of this type would have fatty acids esterified, or present in alkyl linkage, directly to the 5'-hydroxyl of the nucleoside according to the synthetic 20 method of Example 13.

Alternatively, a "spacer" molecule having, for example, carboxyl groups at either end and 0 to 10 CH_2 groups in the center, could be esterified to the 5'-hydroxyl of the antiviral nucleoside. The other carboxyl 25 of the "spacer" may be esterified to the free hydroxyl of diacylglycerol or any other lipid having an available hydroxyl function. Other linking ("spacer") groups with suitable functional groups at the ends may also be used to link the diglyceride or other suitable lipid group to the 30 nucleoside, by chemical methods well known to those skilled in the art.

Preparation of Liposomes comprising Lipid Derivatives of Antiviral Nucleosides

After synthesis, the lipid derivative of the 35 nucleoside analogue is incorporated into liposomes, or other suitable carrier. The incorporation can be carried out according to well known liposome preparation

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procedures, such as sonication, extrusion, or microfluidization. Suitable conventional methods of liposome preparation include, but are not limited to, those disclosed by Bangham, et al. (4), Olson, et al. (26), Szoka 5 and Papahadjopoulos (27), Mayhew, et al. (28), Kim, et al. (29), Mayer, et al. (30) and Fukunaga, et al. (31).

The liposomes can be made from the lipid derivatives of nucleoside analogues alone or in combination with any of the conventional synthetic or natural phospholipid liposome 10 materials including phospholipids from natural sources such as egg, plant or animal sources such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, sphingomyelin, phosphatidylserine, or phosphatidylinositol. Synthetic phospholipids that may 15 also be used, include, but are not limited to, dimyristoylphosphatidylcholine, dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine, and the corresponding synthetic phosphatidylethanolamines and phosphatidylglycerols. Other additives such as 20 cholesterol or other sterols, cholesterol hemisuccinate, glycolipids, cerebrosides, fatty acids, gangliosides, sphingolipids, 1,2-bis(oleoyloxy)-3-(trimethyl ammonio)propane (DOTAP), N-[1-(2,3-dioleoyl) propyl]-N,N,N-trimethylammonium (chloride) (DOTMA), D,L,-2,3- 25 distearoyloxypropyl(dimethyl)- β -hydroxyethyl ammonium (acetate), glucopsychosine, or psychosine can also be added, as is conventionally known. The relative amounts of phospholipid and additives used in the liposomes may be 30 varied if desired. The preferred ranges are from about 80 to 95 mole percent phospholipid and 5 to 20 mole percent psychosine or other additive. Cholesterol, cholesterol hemisuccinate, fatty acids or DOTAP may be used in amounts ranging from 0 to 50 mole percent. The amounts of 35 antiviral nucleoside analogue incorporated into the lipid layer of liposomes can be varied with the concentration of

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their lipids ranging from about 0.01 to about 100 mole percent.

Using conventional methods to entrap active compound entraps approximately 20 to 50% of the material present in 5 solution; thus, approximately 50 to 80% of the active compound is wasted. In contrast, where the nucleoside analogue is incorporated into the lipids, virtually all of the nucleoside analogue is incorporated into the liposome, and virtually none of the active compound is wasted.

10 The liposomes with the above formulations may be made still more specific for their intended targets with the incorporation of monoclonal antibodies or other ligands specific for a target. For example, monoclonal antibodies to the CD4 (T4) receptor may be incorporated into the 15 liposome by linkage to phosphatidylethanolamine (PE) incorporated into the liposome by the method of Leserman, et al. (19). As previously described, HIV will infect those cells bearing the CD4 (T4) receptor. Use of this CD4-targeted immunoliposome will, therefore, focus 20 antiviral compound at sites which HIV might infect. Substituting another CD4 recognition protein will accomplish the same result. On the other hand, substituting monoclonal antibody to gp110 or gp41 (HIV viral coat proteins) will focus antiviral immunoliposomes 25 at sites of currently active HIV infection and replication. Monoclonal antibodies to other viruses, such as Herpes simplex or cytomegalovirus will focus active compound at sites of infection of these viruses.

Therapeutic Uses of Lipid Derivatives

30 The liposome incorporated phosphorylated nucleoside analogue is administered to patients by any of the known procedures utilized for administering liposomes. The liposomes can be administered intravenously, intraperitoneally, intramuscularly, or subcutaneously as a 35 buffered aqueous solution. Any pharmaceutically acceptable aqueous buffer or other vehicle may be utilized so long as

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it does not destroy the liposome structure or the activity of the lipid nucleoside analogue. One suitable aqueous buffer is 150 mM NaCl containing 5 mM sodium phosphate with a pH of about 7.4 or other physiological buffered salt 5 solutions.

The dosage for a mammal, including a human, may vary depending upon the extent and severity of the infection and the activity of the administered compound. Dosage levels for nucleoside analogues are well established. Dosage 10 levels of lipid derivatives of nucleoside analogues should be such that about 0.001 mg/kilogram to 1000 mg/kilogram is administered to the patient on a daily basis and more preferably from about 0.05 mg/kilogram to about 100 mg/kilogram.

15 The present invention utilizes the antiviral nucleoside derivatives noted above incorporated in liposomes in order to direct these compounds to macrophages, monocytes and any other cells which take up the liposomal composition. Ligands may also be 20 incorporated to further focus the specificity of the liposomes.

The derivatives described have several unique and novel advantages over the water soluble dideoxynucleoside phosphates described in an earlier copending application.

25 First, they can be formulated more efficiently. Liposomes comprising lipid derivatives of nucleoside analogues have much higher ratios of drug to lipid because they are incorporated into the wall of the liposome instead of being located in the aqueous core compartment. 30 Secondly, the liposomes containing the lipophilic dideoxynucleoside derivatives noted above do not leak during storage, providing improved product stability. Furthermore, these compositions may be lyophilized, stored dry at room temperature, and reconstituted for use, 35 providing improved shelf life. They also permit efficient incorporation of antiviral compounds into liposomal

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formulations without significant waste of active compound.

They also provide therapeutic advantages. Stability of the liposomally incorporated agent causes a larger percentage of the administered antiviral nucleoside to 5 reach the intended target, while the amount being taken up by cells in general is minimal, thereby decreasing the toxic side effects of the nucleosides. The toxic side effects of the nucleosides may be further reduced by targeting the liposomes in which they are contained to 10 actual or potential sites of infection by incorporating ligands specifically binding thereto into the liposomes.

Finally, the compounds noted above have been constructed in a novel way so as to give rise to phosphorylated dideoxynucleosides or other antiviral 15 nucleosides upon further cellular metabolism. This improves their antiretroviral (antiviral) effect in monocytes and macrophages or other cells which are known to be resistant to the effects of the free antiviral compounds. Further, the compounds pre-incubated with 20 lymphoid cells provide complete protection from HIV infection for up to and exceeding 48 hours after the drug is removed, while the free nucleoside provides no protection 24 hours after removal. Finally, the lipid compounds are expected to be useful in treating HIV 25 infections due to strains of virus which are resistant to free antiretroviral nucleoside analogues.

Lipid derivatives of antiviral agents have a prolonged antiviral effect as compared to the lipid-free agents; therefore they provide therapeutic advantages as 30 medicaments even when not incorporated into liposomes. Non-liposomal lipid derivatives of antiviral nucleoside analogues may be applied to the skin or mucosa or into the interior of the body, for example orally, intratracheally or otherwise by the pulmonary route, enterally, rectally, 35 nasally, vaginally, lingually, intravenously, intra-arterially, intramuscularly, intraperitoneally,

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intradermally, or subcutaneously. The present pharmaceutical preparations can contain the active agent alone, or can contain further pharmaceutically valuable substances. They can further comprise a pharmaceutically acceptable carrier.

5 Pharmaceutical preparations containing lipid derivatives of antiviral nucleosides are produced by conventional dissolving and lyophilizing processes to contain from approximately 0.1% to 100%, preferably from 10 approximately 1% to 50% of the active ingredient. They can be prepared as ointments, salves, tablets, capsules, powders or sprays, together with effective excipients, vehicles, diluents, fragrances or flavor to make palatable or pleasing to use.

15 Formulations for oral ingestion are in the form of tablets, capsules, pills, ampoules of powdered active agent, or oily or aqueous suspensions or solutions. Tablets or other non-liquid oral compositions may contain acceptable excipients, known to the art for the manufacture 20 of pharmaceutical compositions, comprising diluents, such as lactose or calcium carbonate; binding agents such as gelatin or starch; and one or more agents selected from the group consisting of sweetening agents, flavoring agents, coloring or preserving agents to provide a palatable 25 preparation. Moreover, such oral preparations may be coated by known techniques to further delay disintegration and absorption in the intestinal tract.

30 Aqueous suspensions may contain the active ingredient in admixture with pharmacologically acceptable excipients, comprising suspending agents, such as methyl cellulose; and wetting agents, such as lecithin or long-chain fatty alcohols. The said aqueous suspensions may also contain preservatives, coloring agents, flavoring agents and sweetening agents in accordance with industry standards.

35 Preparations for topical and local application comprise aerosol sprays, lotions, gels and ointments in

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pharmaceutically appropriate vehicles which may comprise lower aliphatic alcohols, polyglycols such as glycerol, polyethylene glycol, esters of fatty acids, oils and fats, and silicones. The preparations may further comprise 5 antioxidants, such as ascorbic acid or tocopherol, and preservatives, such as p-hydroxybenzoic acid esters.

PARENTERAL PREPARATIONS comprise particularly sterile or sterilized products. Injectable compositions may be provided containing the active compound and any of the 10 well known injectable carriers. These may contain salts for regulating the osmotic pressure.

The therapeutically effective amount of the lipid derivatives is determined by reference to the recommended dosages of the active antiviral nucleotide, bearing in 15 mind that, in selecting the appropriate dosage in any specific case, consideration must be given to the patient's weight, general health, metabolism, age and other factors which influence response to the drug. The parenteral dosage will be appropriately an order of magnitude lower 20 than the oral dose.

A more complete understanding of the invention can be obtained by referring to the following illustrative examples, which are not intended, however, to unduly limit the invention.

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EXAMPLE 1

Synthesis of

5 1,2-Dimyristoylglycerophospho-5'-(3'-azido-3'-
deoxy)thymidine, monosodium salt.

Preparation of dimyristoylphosphatidic acid (DMPA-H):

In a separatory funnel (500 ml), dimyristoylphosphatidic acid disodium salt (1 g., 1.57 mmol) was first dissolved in chloroform:methanol (2:1 by volume, 250 ml) and mixed well. Distilled water (50 ml) was added to the solution, and the pH was adjusted to 1 by adding concentrated hydrochloric acid. The solution was mixed well and the chloroform layer collected. The 15 chloroform layer was back washed once with methanol:water (1:1 by volume, 80 ml) and evaporated under reduced pressure at 30°C to yield dimyristoylphosphatidic acid (DMPA-H) as a white foam. Cyclohexane (10 ml) was added and the solution lyophilized to dryness to obtain a white 20 powder (850 mg) which was then stored at -20°C. A day before the coupling reaction, DMPA-H (250 mg, 0.42 mmol) was dissolved in cyclohexane (10 ml) in a round-bottom (50 ml) flask and the solvent evaporated under reduced pressure at room temperature. This process was repeated four more 25 times and the DMPA-H further dried in the vacuum oven at room temperature overnight over P₂O₅ and stored in a desiccator at -20°C.

Coupling reaction:

30 Under argon, to the 50 ml round-bottom flask containing dried DMPA-H (250 mg, 0.42 mmol), dried 3'-azido-3'-deoxythymidine (AZT), Sigma Chemical, St. Louis, Missouri, (85 mg, 0.31 mmol, dried over P₂O₅ under vacuum overnight), and 2,4,6-triisopropylbenzenesulfonyl chloride 35 (315 mg, 1.04 mmol) was added, and anhydrous pyridine (2 ml) added via syringe to obtain a clear solution. The reaction mixture was stirred at room temperature for 18 hours. (The reaction was followed by thin layer

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chromatography). Water (1 ml) was added to the crude product to destroy excess catalyst and the solvent was evaporated under reduced pressure to yield a yellow gum which was then redissolved in a small volume of 5 methanol:chloroform (1:9 by volume) and applied to a column of silica gel (45 g, Kieselgel 60, West Germany). The column was eluted with 8% methanol in chloroform. After a forerun (rejected), AZT was recovered, and then dimyristoylphosphatidyl-3'-azido-3'-deoxythymidine (DMPA-AZT) was obtained. The fractions containing the product 10 were combined and the solvent was evaporated under reduced pressure. Cyclohexane (5 ml) was added to the residue and the mixture lyophilized to dryness under vacuum over P₂O₅ to yield pure DMPA-AZT (270 mg, 0.29 mmol, 95%).

15

Conversion to monosodium salt:

To the dried DMPA-AZT redissolved in chloroform:methanol (2:1 by volume, 30 ml), distilled water (6 ml) was added, mixed well, and the pH of the aqueous 20 layer was adjusted to 1. The chloroform layer was collected and 10 ml of methanol:water (1:1,) was added and mixed well. The pH of the aqueous layer was adjusted to 6.8 with methanolic NaOH (0.1N N), mixed well, and the aqueous layer was maintained at pH 6.8. The combined 25 chloroform, methanol and water mixture was evaporated under reduced pressure to yield dimyristoylphosphatidyl 3'-azido-3'-deoxythymidine monosodium salt. The residue was redissolved in chloroform:methanol (2:1 by volume, 2 ml) and acetone added to precipitate DMPA-AZT monosodium salt 30 which was further dried from cyclohexane (5 ml) to yield a white powder (220 mg, 0.26 mmol, 78% yield based on AZT). The melting point was 230°C; R_f value on silica gel G thin layer plates was 0.32 (chloroform:methanol:water:ammonia 80:20:1:1), R_f 0.58 (chloroform:methanol:water:ammonia 35 70:30:3:2), R_f 0.31 (chloroform:methanol:water 65:25:4); UV absorption maximum 266nm (ϵ 10,800); Analysis Calculated

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for C₄₁N₅O₁₁P₁H₇₂. 1 H₂O: C, 57.24; H, 8.44; P, 3.61; Found: C, 56.80; H, 8.83; P, 3.52. MS, m/e 864.60 (M+)

Proton NMR: (CDCl₃) δ 0.88 (6H, bt, J=6.9Hz, acyl CH₃), 1.26 (40H, s, acyl CH₂), 1.60 (4H, bs, β acyl CH₂), 1.94 5 (3H, s, thymine CH₃), 2.31 (4H, m, α acyl CH₂), 2.39 (2H, m, ribose 2'H), 3.38 (2H, bd, J=12.6Hz, ribose 5'H), 3.78 (2H, m, sn-3 CH₂ glycerol), 4.00 (1H, dd, J₁=12Hz, J₂=6Hz, sn-1 CH₂ glycerol), 4.18 (1H, dd, J₁=12Hz, J₂=6Hz, sn-1 10 CH₂ glycerol), 4.07 (1H, m, ribose 3'H), 4.41 (1H, m, ribose 4'H), 5.24 (1H, m, sn-2 CH glycerol), 7.62 (1H, s, thymine 6H), 6.21 (1H, t, J=6Hz, ribose 1'H). The peak area ratio of phosphatidic acid to AZT is 1.

EXAMPLE 2

3'-deoxythymidine was obtained from Sigma Chemical, St. Louis, Missouri. The lipid derivative of this analogue was synthesized using the same method described above in Example 1. Melting Point 235°C, Rf on silica gel G 0.25 (chloroform/methanol/water/ammonia 80:20:1:1); 0.57 (chloroform:methanol:ammonia:water 70:30:3:2); 0.24 (chloroform:methanol:water 64:25:4); UV absorption maximum 269 nm (ϵ 8,400); Analysis: Calculated for C₄₁N₂O₁₁P₁H₇₂Na₁·1H₂O: C, 58.53; H, 8.87; P, 3.69; Found: C, 56.75; H, 9.33; P, 3.58. MS, m/e 823.00 (M⁺).

30 Proton NMR: (CDCl₃) δ 0.91 (6H, bt, J=6.8Hz, acyl CH₃),
 1.23 (4H, bs, acyl CH₂), 1.26 (4H, bs, acyl CH₂), 1.28
 (32H, bs, acyl CH₂), 1.62 (4H, m, β acyl CH₂), 1.97 (3H, s,
 thymine CH₃), 2.05 (2H, m, ribose 2'H), 2.35 (4H, m, α acyl
 CH₂), 3.39 (2H, bs, ribose 5'H), 3.90 (2H, m, sn-1 CH₂
 glycerol), 4.16 (1H, m, sn-1 CH₂ glycerol), 4.24 (1H, m,
 35 sn-1 CH₂ glycerol), 4.38 (1H, m, ribose 4'H), 5.23 (1H, m,
sn-2 glycerol) 6.10 (1H, bt, ribose 1'H), 7.68 (1H, s,

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thymine 6H). The peak area ratio of phosphatidic acid to 2'3'-dideoxythymidine is 1.

5

EXAMPLE 3

Synthesis of 1,2-Dimyristoylglycerophospho-5'-(2',3'-dideoxy)cytidine

Preparation of 4-acetyl-2'3'-dideoxycytidine:

10 To a stirred, refluxing solution of 2'-3'-dideoxycytidine (DDC) (400 mg, 1.89 mmol) in anhydrous ethanol (35 ml, dried first with Lindy type 4x molecular sieve, and twice distilled over magnesium turnings) was added acetic anhydride (0.4 ml, 5.4 mmol). During the 15 course of a 3 hour refluxing period, four more additional 0.4 ml portions of acetic anhydride were added at 30 minute intervals. The reaction was followed by thin layer chromatography (silica gel F254, Kodak Chromagram, developed with 10% methanol in chloroform). After the 20 final addition, the solution was refluxed for 1 more hour. The reaction mixture was cooled and solvent was evaporated under diminished pressure. The residue was redissolved in 8% methanol in chloroform (5 ml) and chromatographed on a silica gel column (2.2 cm x 30 cm, Kieselgel 60, 70-230 25 mesh, EM Science, 45 g). The column was eluted with 8% methanol in chloroform to yield pure 4-acetyl-2'3'-dideoxycytidine (DDC-OAC) in 80% yield.

Coupling reaction:

30 A day before the coupling reaction, DMPA-H (prepared as before, 250 mg, 0.42 mmol) was dissolved in cyclohexane (10 ml) in a round-bottom flask (50 ml) and the solvent evaporated under reduced pressure at room temperature. This process was repeated four more times and DMPA-H 35 further dried in a vacuum oven at room temperature overnight over P₂O₅. Under argon, to the 50 ml round-bottom flask containing dried DMPA-H was added dried (DDC-

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OAC) (85 mg, 0.33 mmol, dried over P_2O_5 under vacuum overnight), and 2,4,6-triisopropylbenzenesulfonyl chloride (315 mg, 1.04 mmol), and anhydrous pyridine (2 ml) via syringe to obtain a clear solution. The reaction mixture 5 was stirred at room temperature for 18 hours. (The reaction was followed by thin layer chromatography). Water (1 ml) was added to the mixture to destroy excess catalyst. The solvent was evaporated under reduced pressure to yield a yellow gum which was redissolved in a small volume of 10 methanol in chloroform (1:9 by volume) and applied to a column of silica gel (45 g, Kieselgel 60, EM Science). The column was topped with a small amount of sand (500 mg) to prevent the sample from floating during elution. The column was eluted with 8% methanol in chloroform (1.5L). 15 After a forerun (rejected), then dimyristoylphosphatidyl-5'-(2'3'-dideoxy)cytidine (DMPA-DDC) was obtained. The fractions containing the product were combined and the solvent was evaporated under reduced pressure. The residue was further dried with cyclohexane to yield pure DMPA-DDC- 20 OAC (210 mg, 0.21 mmol, in 70% yield). R_f 0.40 (silica gel GF, 20x20 cm, Analtech, chloroform:methanol:water: ammonia 80:20:1:1 by volume).

Deblocking with 9N NH4OH:

25 DDC-OAC-DMPA (40 mg, 0.04 mmol) was dissolved in chloroform:methanol (1:1, 2 ml), and 9N NH_4OH (10 drops) was added at once. The solution was stirred at room temperature for 15 minutes and was then quickly neutralized with glacial acetic acid to pH 7. The neutralized solution 30 was evaporated to dryness overnight under reduced pressure to yield dimyristoylphosphatidyl 5'-(2'3'-dideoxy)cytidine (DMPA-DDC, 35 mg, 0.037 mmol). Melting point: DMPA-ddC decomposed at 240°C. On thin layer chromatography on 35 silica gel GF plates, the R_f values were: 0.11 (chloroform:methanol:water:ammonia 80:20:1:1); 0.38 (chloroform:methanol:ammonia:water 70:30:3:2); 0.15

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(chloroform:methanol:water 65:25:4); UV absorption maximum 273 nm (ϵ 5,800).

NMR: (CDCL3) δ 0.86 (6H, bt, acyl CH3), 1.24 (40H, bs, acyl CH2), 1.57 (4H, m, β acyl CH2), 2.28 (4H, m, α acyl CH2), 5 3.36 (2H, m, ribose 5'H), 3.94 (2H, bs, sn-3 CH2 glycerol), 4.19 (1H, m, sn-1 CH2 glycerol), 4.29 (1H, m, sn-1 CH2 glycerol), 4.40 (1H, bs, ribose 4'H), 5.19 (1H, m, sn-2 CH glycerol), 5.89 (1H, m, thymine 5-H), 7.44 (1H, bs, thymine NH3), 7.94 (1H, bs, thymine NH2). The peak area 10 ratio of phosphatidic acid to 2'3'-dideoxycytidine is 1.

EXAMPLE 4

Synthesis of
(3'Azido-3'-deoxy)thymidine-5'-diphosphate-sn-3-(1,2-dipalmitoyl)glycerol

15

Synthesis of AZT-monophosphate morpholidate:

This compound was synthesized following the method of Agranoff and Suomi (21). AZT-monophosphate was converted into the acidic form by passing a solution in water through 20 a column of Dowex 50W (50x2-200, 100-200 mesh, Sigma Chemicals, St. Louis, MO). A solution of 117 mg AZT-monophosphate (0.3 millimoles) in 3 ml of water was transferred to a two neck round bottom flask. The 3 ml of t-butanol and 0.106 ml of freshly distilled morpholine 25 (1.20 millimoles) were added and the mixture was placed in a oil bath at 90 °C. Four equivalents of dicyclohexylcarbodiimide 249 mg, 1.20 millimole) in 4.5 ml of t-butanol were added dropwise. The reaction was monitored by thin layer chromatography on silica gel 60, F 30 254, plates (E. Merck, Darmstadt) with chloroform/methanol/acetic acid/water (50/25/3/7 by volume) as the developing solvent. The reaction was noted to be complete after 3 hours. The mixture was cooled and after addition of 4.5 ml of water was extracted four times with 35 15 ml of diethylether. The aqueous layer was evaporated to dryness and dried in vacuo over P2O5. The product was obtained (199 mg, 100% yield) and used for coupling to phosphatidic acid without further purification.

Coupling of AZT-monophosphate morpholidate to dipalmitoylphosphatidic acid:

Dipalmitoylphosphatidic acid, disodium salt was 5 converted to the free acid by extracting the material from chloroform by the method of Bligh and Dyer (34) using 0.1N HCl as the aqueous phase. The chloroform layer was 10 evaporated to dryness in vacuo, the phosphatidic acid (196 mg, 0.3 millimoles) was redissolved in chloroform and transferred to the vessel containing the AZT-monophosphate morpholidate. After the chloroform was 15 removed in vacuo using a rotary evaporator, the mixture was dried by addition and evaporation of benzene and finally dried in vacuo over P_2O_5 . The reaction was started by 20 addition of 30 ml of anhydrous pyridine and the clear mixture was stirred at room temperature. The reaction was monitored with thin layer chromatography as noted above with chloroform/methanol ammonia/water (70/38/82 by volume) as developing solvent. The Rf of phosphatidic acid, AZT-monophosphate morpholidate and AZT-diphosphate 25 dipalmitoylglycerol is 0.11, 0.50, and 0.30, respectively.

After 70 hours the pyridine was evaporated and the product was extracted into chloroform after addition of 15 ml of water, 30 ml of methanol, 22 ml chloroform and 25 sufficient 1N formic acid to adjust the pH to 4.0. The combined chloroform layers after two extractions were evaporated to dryness, the residue was dissolved in chloroform/methanol/ammonia/water, 70/38/8/2, and the product was purified by silica gel column chromatography in 30 this solvent applying an air pressure equivalent to one meter of water. Fractions not completely pure were further purified by HPLC on a reverse phase column (Vydac C18) using water/methanol (8/2 by volume) and methanol as 35 eluents. Fractions containing the desired product were evaporated to dryness to give 132 mg. of a white solid (44% yield) which gave a single spot by thin layer

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chromatography with silica gel g plates developed with chloroform/methanol/ammonia/water, 70/38/8/2 (Rf 0.35) and chloroform/methanol/water, 65/35/4 (Rf 0.54).

500 MHz NMR (CDCl₃) δ 0.88 (3H, t, J=6.93 Hz, sn-2-acyl CH₃), 0.92 (3H, t, J=7.48 Hz, sn-1-acyl chain CH₃), 1.25 (48H, s, CH₂ acyl chains), 1.55 (4H, bs, β CH₂ acyl chains) 1.83 (3H, s, CH₃ thymine), 2.25 (2H, t, J=6.97 Hz, 2H, alpha CH₂ sn-2-acyl chain), 2.27 (2H, t, J=7.79 Hz, α CH₂ sn-1-acyl chain), 2.44 (4H, bs, 2' and 5' H ribose), 3.78 10 (1H, dd, J=1.68, 5.51 Hz, 3'H ribose), 3.95 (2H, bs, sn-3 CH₂ glycerol), 4.07 (1H, bs, H_e/H_a sn-1 CH₂ glycerol), 4.13 (1H, bs, 1H, sn-2 CH glycerol), 4.36 (1H, bs, H_a/H_e sn-1 CH₂ glycerol), 5.21 (1H, bs, sn-2 CH glycerol), 5.66 (1H, bs, 1'H ribose), 7.14 (1H, d, J=6.25 Hz, 6H thymine). The 15 ratio of acyl chains: glycerol:ribose: thymine as deduced from appropriate resonances amounted to 2.12:0.93:0.98:1.00. IR (KBr, disk) showed 2105 (azido), 1745 (c=o ester) and 1705 (c=o thymine) as identifiable bands.

20

EXAMPLE 5

Synthesis of an antiviral nucleoside diacyl phosphate

Dihexadecyl phospho-5'-dideoxycytidine is synthesized according to the method described in Example 1, except that 25 the reactants are dideoxycytidine and dihexadecyl hydrogen phosphate. The starting material dihexadecyl hydrogen phosphate is synthesized from hexadecan-1-ol and phenyl phosphorodichloride as first reported by D. A. Brown, et al. (32).

30

EXAMPLE 6

Synthesis of Dideoxyadenosine diphosphate ceramide an antiviral phosphonucleoside

The method of Example 2 is repeated, except that 35 dideoxyadenosine monophosphate morpholidate is substituted for the dideoxycytidine monophosphate morpholidate. Ceramide phosphoric acid is prepared by the action of

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phosphorus oxychloride on ceramide. Ceramide phosphoric acid is substituted for the dimyristoyl phosphatidic acid. Similar results are obtained.

5

EXAMPLE 7

Synthesis of
1-0-stearoylglycero-rac-3-phospho-5'-(3'-deoxy,
3,-azido)thymidine:

10 Dry 1-0-stearoyl-rac-3-glycerol (batyl alcohol, 250 mg), 3'-azido-3'deoxythymidine monophosphate sodium salt (0.725 gm) and 2,4,6,-triisopropylbenzenesulfonyl chloride (TPS, 1.219 gm) were mixed in dry pyridine and stirred overnight under nitrogen. Chloroform (50 ml) was added and
15 the reaction mixture was washed twice with cold 0.2N HCl and 0.2N sodium bicarbonate. The organic phase was removed in vacuo with a rotary evaporator and the product was crystallized at -20°C from 20 ml of chloroform/acetone (12:8 by volume). The final purification of the compound
20 was done by preparative thin layer chromatography using 500 micron layers of silica gel G developed with chloroform/methanol/concentrated ammonia/water (70/30/1/1 by volume).

25 In the preceding syntheses, proton NMR spectra were obtained with a General Electric QE-300 spectrometer, using tetramethylsilane as internal standard (key: s=singlet, d=doublet, t=triplet, q=quartet, dd=doublet of doublets, b=broad), UV spectra were recorded on Shimadzu UV-160,
30 spectrophotometer. Fast atom bombardment mass spectra were determined by Mass Spectrometry Service Laboratory, University of Minnesota. Elemental analyses were determined by Galbraith Laboratories, Knoxville, TN. and Schawarzkopf Microanalytical Laboratory, N.Y. Melting
35 points were obtained with a Fisher-Johns melting apparatus. Column chromatography was carried out on Merck silica gel 60 (70-230 mesh). Rf values were obtained with HPTLC

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Merck, Kieselgel 60 pre-coated plates, 10x10cm. Anhydrous pyridine, 2,4,6-Triisopropylbenzenesulfonyl chloride (TPS), and 3'-azido-3'-deoxythymidine (AZT) were purchased from Aldrich. Dimyristoylphosphatidic acid, disodium salt, was 5 purchased from Avanti; batyl alcohol was obtained from Sigma Chemical, St. Louis, Missouri.

EXAMPLE 8

10 Preparation of Liposomes containing
Antiretroviral Liponucleotides

6.42 micromoles of dioleoylphosphatidylcholine, 3.85 micromoles of cholesterol, 1.28 micromoles of dioleoylphosphatidylglycerol and 1.28 micromoles of 15 dimyristoylphosphatidyl-azidothymidine were mixed in a sterile 2.0 ml glass vial and the solvent was removed in vacuo in a rotary evaporator. In some experiments, dimyristoylphosphatidylazidothymidine was replaced with either dimyristoylphosphatidylideoxythymidine, 20 dimyristoylphosphatidylideoxycytidine or azidothymidine diphosphate dimyristoylglycerol; control liposomes were prepared by omitting the antiviral liponucleotide. The dried film was placed under high vacuum overnight at room temperature to remove traces of solvent. The lipid film 25 was hydrated at 30°C with 0.3 ml of sterile 10 mM sodium acetate buffer (pH 5.0) containing isotonic dextrose and the ampule was sealed. The mixture was vortexed intermittently for 10 minutes followed by sonication using a Heat Systems Ultrasonics sonicator with a cup horn 30 generator (431B) at output control setting #9 for 90 to 120 minutes at which time the sample is clarified. This sonicated preparation was diluted with sterile RPMI buffer and added to the tissue culture wells at the concentration indicated.

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EXAMPLE 9

**Coupling of monoclonal antibodies to CD4
to an antiviral lipid-containing liposome**

5 Dimyristoylphosphatidyl-AZT produced by the method of Example 1, dimyristoylphosphatidylcholine, cholesterol and dimyristoylphosphatidylethanolamine in a molar ratio of 39:39:20:2. 200 mg of this lipid mixture was dried in vacuo using a rotary evaporator to form a thin film in a
10 100 ml round-bottom flask. 1 ml of sterile phosphate buffered saline was added and the mixture shaken gently at 20°C. for 20 minutes, followed by ten 30-second cycles of vortexing to form multilamellar liposomes. The suspension was subjected to 5 cycles of extrusion through two stacked
15 Nucleopore polycarbonate filters having pore diameters of 200 nm to produce a homogeneous liposomal population. Other methods may be used such as sonication, reverse phase evaporation and use of a French press or Microfluidizer (Microfluidics, Newton, Massachusetts). 1 to 2 mg of OKT4a
20 monoclonal antibodies to CD4 antigen are thiolated by incubation with 0.08 mM N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP). Untreated SPDP is removed by gel filtration through Sephadex G25. The voiding DTP-protein is reduced with 0.05 M dithiothreitol
25 in 0.1 M acetate buffered saline at pH 4.5 for 20 minutes, producing reduced thiolated antibody.

Liposomes produced by the method of Example 5, representing 5 micromoles of phospholipid are incubated overnight at room temperature with 1 mg of thiolated antibody in 0.20 ml of isotonic MES/HEPES buffer, pH 6.7. The resulting immunoliposomes are purified by the discontinuous metrizamide gradient method of Heath et al. (33) and sterilized by passage through 200 nm filters.

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EXAMPLE 10

Inhibition of HIV Replication in Tissue Culture Cells
by Lipid Nucleoside Conjugates

5 A. METHODS

Viral infection of Human T-cells:

The human T lymphoblastoid cell line, CCRG-CEM (hereafter referred to as CEM), was grown in RPMI 1640 medium containing 100 U/ml penicillin G, 100 ug/ml 10 streptomycin, 2 mM glutamine and 10% fetal bovine serum (Hyclone Laboratories, Logan, Utah). Cells were infected with the LAV-1 strain (L. Montagnier, Paris, France) at a multiplicity of infection of one tissue culture 50% infectious dose (TCID₅₀)/cell for 60 minutes at 37°C in 15 medium containing 1% polybrene. CEM cells were infected in suspension at 6 X 10⁴ cells/ml, washed three times by centrifugation and resuspension and then distributed in 96-well plates at 6 X 10⁴ cells/well before addition of medium 20 containing the liposomal antiretroviral liponucleotide drugs.

Antiviral Activity as determined by HIV p24 Assay:

Antiviral activity was assayed after 3 days by the inhibition of the production of HIV p24 (gag) antigen in the cell free culture medium of the infected cells exposed 25 to different concentrations of drug; p24 antigen was measured by ELISA (Abbott Laboratories, Chicago, IL) according to the manufacturer's instructions. The data are the average of two determinations and are expressed as percentage of a control incubated in the absence of drugs.

30

B. Experiment H533-1: Figure 1

Liposomes containing 10 mole percent of either dimyristoylphosphatidylazidothymidine (LN1), dimyristoylphosphatidylideoxythymidine (LN2) or 35 azidothymidine diphosphate dimyristoylglycerol (LN4) in the indicated concentrations were tested for their ability to inhibit HIV replication in CEM (wild type) cells in

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vitro. All three of these antiretroviral liponucleotides inhibited HIV p24 production; the amounts of drug required to reduce virus production by 50% (E.D. 50) were as follows:

5

Phosphatidylazidothymidine (LN1)	2 uM
Phosphatidyldeoxythymidine (LN2)	30 uM
AZT diphosphate dimyristoylglycerol (LN4)	8 uM

10

This demonstrates that the lipid derivatives of antiretroviral nucleotides can enter CEM cells and be converted to active nucleoside as predicted. The control liposomes (CONT) which did not contain any antiretroviral 15 nucleotide had no effect on p24 production by CEM cells.

C. Experiment H747-1a: Figure 2

Dimyristoylphosphatidylazidothymidine in liposomes (LN1) was compared with free azidothymidine (N1). At low 20 concentrations below 0.1 uM free AZT was more effective than the liponucleotide. At concentrations ranging from 2 to 170 uM the phosphatidylAZT liposomes were more effective than the free AZT. Control liposomes (CONT) containing only inactive lipids as noted in methods were ineffective in 25 reducing p24.

D. Experiment H747-1b: Figure 3

Dideoxythymidine (N2) is a weak inhibitor of HIV p24 production. Surprisingly, phosphatidyldeoxythymidine 30 (LN2) is somewhat more effective than the free nucleoside. As can be seen in the chart, slightly more free ddT is required to reduce p24 production than with phosphatidyldeoxythymidine. Control liposomes (CONT) at a matched total phospholipid concentration are without 35 effect.

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E. Experiment H637-1b: Figure 4

In this experiment, CEM cells were replaced with mutant cells (provided by Dr. Dennis Carson, Scripps Clinic, San Diego, CA) which lack the thymidine kinase enzyme (CEM tk-). These cells are unable to phosphorylate thymidine derivatives and AZT is therefore inactive since it cannot be converted to the active triphosphate derivative which is needed to inhibit HIV p24 replication. As shown in the chart, AZT (N1) is completely without effect on p24 production over a wide range of concentrations (0.2 to 100 μ M). However, both phosphatidylAZT (LN1) and phosphatidylddT (LN2) were capable of reducing p24 production, proving that these compounds are metabolized in the cell to the nucleoside-monophosphate which can be further activated to the triphosphate by other cellular enzymes. This data provides proof of the principles outlined in the patent which predict direct metabolism to the nucleoside monophosphate.

F. Experiment H805-1: Figure 5

In this experiment dimyristoylphosphatidyl-dideoxycytidine (LN3) and dimyristoyldideoxythymidine (LN2) were compared with the effects of free AZT (N2) and dideoxycytidine (N3) in CEM (wild type) cells in vitro. PhosphatidylddC was the most potent liponucleotide (ED₅₀ 1.1 μ M) and phosphatidylddT was less active as noted before (ED₅₀ 20 μ M). Free liposomes without added antiretroviral nucleotide (CONT) were inactive.

G. Experiment I276:

In this experiment, antiviral protection provided by preincubation with dimyristoylphosphatidylazidothymidine (LN1) in liposomes prepared as noted above was compared with that of free azidothymidine (N1). CEM (wild type) cells were preincubated for 3 days under standard conditions in RPMI media containing 7.14 μ M of either free

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AZT (N1) or phosphatidylAZT (LN1). The cells were then washed twice with PBS, and fresh RPMI media added. Each group of cells was then divided into three batches. One batch was immediately infected with HIV, as noted above; 5 after washing away unattached HIV, the sample was allowed to incubate in media alone for 3 days. Two other batches were allowed to incubate in media alone for either 24 or 48 hours to allow any intracellular antiviral agent present to become depleted. Then they were infected with HIV, the 10 cells washed free of virus, and fresh RPMI media added. After 3 days of further incubation, the supernates of all batches were tested for the presence of p24 protein.

15 Control Cells: CEM cells were subjected to HIV infection without preincubation; drug was added following HIV infection as indicated, and the cells were incubated for 3 days.

20 Preincubated Cells: CEM cells were preincubated for 3 days with media containing AZT (N1) or phosphatidyl AZT (LN1); after 3 days the cells were washed, subjected to HIV infection followed by addition of media without drugs. After a further incubation for 3 days, p24 was measured.

RESULTS:

		p24: ng/ml after 3 days incubation
25	<u>CEM Controls: No Preincubation</u>	
	HIV infection only	204; 207
	HIV + 7.14 μ M Azidothymidine (N1)	64; 69
30	HIV + 7.14 μ M PhosphatidylAZT (LN1)	16; 16

		Pre-Infection Interval without Drug	p24: ng/ml after 3 days incubation
35	<u>CEM Preincubated Cells</u>		
	7.14 μ M Azidothymidine (N1)	24 h	404; 433
40		48 h	271; 245
	7.14 μ M PhosphatidylAZT(LN1)	24 h	6; 7
		48 h	4; 15

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After a 3 day preincubation, followed by 48 hours of incubation in normal media after removal of the drugs, phosphatidylAZT provided complete protection from HIV replication as assessed by the reduced p24 production.

5 However, AZT preincubation failed to protect the cells from HIV infection 24 and 48 hours after removal of the drug.

H. Experiment J45:

In this experiment the compound of Example 7 (1-0-stearoylglycero-rac-3-phospho-5'-(3'-deoxy, 3'-azido)thymidine) was incorporated into liposomes containing 10 mole percent of the liponucleotide as indicated in Example 8. This material was diluted with RPMI medium to the desired concentration and added to HT4-6C cells (CD4+ HeLa cells) obtained from Dr. Bruce Chesbro of the Rocky Mountain National Laboratories (Hamilton, Montana) which had been infected with LAV-1 as noted earlier in this example. After a 3 day incubation at 37°C, the cells were washed with PBS, fixed and stained with crystal violet and 20 the plaques were counted. The results are shown below.

	Liponucleotide Concentration	Plaques, Average	% of Untreated Control
25	10 uM	1	2
	3.16	7	13
	1.0	16	29
	0.316	32	58
	0.100	39	71
30	0.0316	43	78
	0	57	-

The data show that 1-0-stearoyl-rac-3-phospho-5'-(3'-deoxy, 3'-azido)thymidine is effective in inhibiting HIV plaque formation in HT4-6C cells infected with LAV-1. The concentration required to produce 50% inhibition is about 0.35 micromolar.

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EXAMPLE 11

HIV Paired Isolate:Antiviral Sensitivity
I.C.50, μ M; HT4-6C Plaque Reduction Assay

5

Methods: HT4-6C cells (CD4+ HeLa cells) were obtained from Dr. Bruce Chesebro, Rocky Mountain National Laboratories, Hamilton, MT), and infected with HIV isolates as noted in Example 10. After a 3 day incubation, the cells were washed, fixed and stained with crystal violet and plaques were counted. Clinical samples of HIV were isolated before AZT therapy (Pre) and 6 to 12 months after AZT treatment (Post). (Richman, D.D., Larder, B., and Darby, G., Manuscript submitted for publication, 1989). Using the HT4-6C plaque reduction assay, the sensitivity of the paired clinical isolates was determined using either AZT, phosphatidylAZT or phosphatidylddT.

	<u>ISOLATE</u>	<u>AZT</u>	<u>p-AZT</u>	<u>pddT</u>
20	<u>A012</u>			
	Pre (G762-3)	0.01	0.53	4.2
	Post (G691-2)	2	0.37	6.6
25	<u>A018</u>			
	Pre (H112-2)	0.01	0.47	7.4
	Post (G910-6)	4	0.59	6.3
30	<u>A036</u>			
	Pre (G174-6c)	0.007	0.42	7.4
	Post (G704-2)	5.6	0.59	4.2
	<u>PCP022</u>			
35	Pre (H112-5)	0.03	0.47	4.2
	Post (G780-1)	5.6	1.05	6.6
	<u>PCP026</u>			
	Pre (H112-6)	0.01	0.33	6.6
	Post (G890-1)	2.8	0.74	2.6

40

Abbreviations: pAZT, phosphatidylazidothymidine; pddt, phosphatidylidideoxythymidine

Post AZT treatment, all 5 isolates showed marked decreases in sensitivity to AZT. This was not observed to

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occur with pAZT and pddT indicating that the post-AZT isolates retain their usual level of sensitivity to the antiretroviral nucleoside administered in the form of novel phospholipid derivatives.

5

EXAMPLE 12

**Synthesis of Phosphatidylacyclovir and
Efficacy in Herpes Simplex Virus-Infected WI-38 Cells**

Dimyristoylphosphatidic acid (disodium salt) was
10 obtained from Avanti Polar Lipids, Birmingham, AL, and
converted to the free acid (DMA-H) as described above in
Example 1. Acycloguanosine (acyclovir, Zovirax^R) was
obtained from Sigma Chemical Co., St. Louis, MO and 73 mg
(0.32 mmol) was dried overnight over phosphorus pentoxide
15 in a vacuum oven. 250 mg of DMPA-H (0.42 mmol) was added
to a 50 ml round bottom flask and dried overnight over
phosphorus pentoxide in a vacuum oven. Under dry argon, 73
mg of acycloguanosine, 315 mg (1.04 mmol) of
triisopropylbenzenesulfonyl chloride (Aldrich, Milwaukee,
20 WI) and 2 ml of dry pyridine (Aldrich, Milwaukee, WI) were
added to the round bottom flask. The reaction mixture was
stirred at room temperature for 18 hours followed by the
addition of 1 ml of distilled water.

The solvent was evaporated in vacuo to yield a yellow
25 gum which was redissolved in a small volume of
chloroform/methanol (9/1) and applied to a column of silica
gel (45 gm: Kieselgel 60, EM Science, Cherry Hill, NJ).
The column was eluted with 8% methanol in chloroform (500
ml), 10% methanol in chloroform (250 ml) followed by 15%
30 methanol in chloroform (1.5 L). After a 1.5 liter forerun
rejected), dimyristoylphosphatidylacyloguanosine (pACV)
was obtained. Three fractions were collected and analyzed:
fraction 1 (200 ml, 130 mg pACV) contained pure pACV;
fraction 2 (200 ml, 150 mg) and fraction 3 (200 ml, 50 mg)
35 contained pACV and small amounts of starting material as
impurities. Fraction 1 was concentrated in vacuo and to
the residue was added 5 ml of cyclohexane; the solution was

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frozen and lyophilized to dryness under phosphorus pentoxide to yield pure phosphatidylcycloguanosine (80 mg, 0.1 mmol).

The purified compound gave a single spot with an Rf of 5 0.29 when applied to K6G silica gel plates (Whatman International, Maidstone, England) developed with chloroform/methanol/water/ammonia (70/30/1 by volume). The UV absorption was maximal at 256 nm (extinction coefficient = 8.4×10^3 in CHCl_3). The percentage phosphorus was 3.30% 10 (theoretical 3.89%) and the melting point was 245°C. On HPLC analysis, phosphatidylcycloguanosine gave a single peak with a retention time of 11 minutes (Spheri-5; Brownlee Labs, Applied Biosystems, Santa Clara, CA) when eluted with a mobile phase of 1-propanol/0.25 mM potassium phosphate/hexane/ethanol/acetic acid (245/179/31/50/0.5 by 15 volume) at a flow rate of 0.5 ml/min.

Cell Cultures

Wi-38 cells were obtained from American Type Culture 20 Collection (Rockville, Maryland 20852) and grown in Dulbecco's minimum essential medium (DMEM) with 10% fetal calf serum (FCS). The cells were grown in 250 cm square bottles until reaching confluence.

25 Virus

Herpes simplex virus (HSV) type 1 (HSV-1) and type 2 (HSV-2) were obtained from the American Type Culture Collection. Both virus stocks were prepared in Wi-38 cells; extensive cytopathic effects (CPD) were observed 30 when the stock virus was harvested by a single freezing and thawing and the cell debris was clarified by low speed centrifugation (2000 rpm). Supernatant fluids containing the virus were aliquoted into small vials and stored at-80C. Both HSV-1 and HSV-2 stocks were titered in Wi-38 35 cells before use in the experiments.

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Herpes Simplex Virus Plaque Reduction Assay

The plaque reduction assay was used to measure the antiviral effect of phosphatidylacyclovir or free ACV. Wi-38 cells were trypsinized with 0.25% trypsin for 5 min. 5 The cells were harvested and centrifuged to remove residual trypsin and the cell pellet was resuspended in DMEM with 10% FCS. The Wi-38 cells were plated in a 96 well plate (5x10 cells/well) for one hour. The infected cells were then treated with phosphatidylacyclovir or ACV. The 10 antiviral agents were prepared in stock solutions which were then diluted two-fold with 2% FBS in DMEM containing 0.5% methylcellulose. 100 μ l of each diluted antiviral agent was added into each well of HSV infected cells.

The control and drug-treated cell cultures were 15 incubated in a 37°C incubator with 5% carbon dioxide for 24 hours. When HSV-infected cells (control without antiviral agent) showed readable number of plaques, the entire plate was fixed with methanol and stained with 1% crystal violet for 10 min. The dye was rinsed off with tap water and the 20 plate was dried and plaques were counted. The antiviral effect of ACV or phosphatidylacyclovir was determined by measurement of plaque reduction as shown in the example below.

25 RESULTS: EFFECT OF ACYCLOVIR AND PHOSPHATIDYLACYCLOVIR
ON PLAQUE FORMATION BY HSV-1 IN WI-38 CELLS

	Acyclovir conc	1	2	mean	% no Drug
30					
	10 μ M	0	0	0	0
	5	0	0	0	0
	2.5	0	0	0	0
35	1.25	4	3	3.5	13
	0.625	8	6	7	26
	0.31	17	19	20	65
	0.155	18	22	20	73
40	0	20;30	30;30	27.5	100

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	PhosphatidylACV	1	2	mean	% no Drug
5	214 uM				
	107	toxic	toxic	-	-
	54	0	0	0	0
	27	2	3	2.5	9
	13.4	4	6	5	18
	6.7	6	9	7.5	27
10	3.3	10	12	11	40
	1.67	17	20	18.5	67
	0.84	24	26	25	91
	0	20;30	30;30	27.5	100

15 The data shown above indicate that phosphatidylacyclovir is effective in HSV-1 infected Wi-38 cells. the concentration which produces 50% inhibition is 2 uM versus 0.4 uM for acyclovir. Similar results were obtained with HSV-2 in infected Wi-38 cells.

20

EXAMPLE 13

Synthesis of 5'-palmitoyl(3'-deoxy-3'-azido)thymidine

0.5 grams of AZT (1.87 mmol) was dissolved in 10 ml of dry chloroform and 2 ml of dry pyridine. 0.78 grams (2.8 mmol) of palmitoyl chloride (Aldrich Chemicals, Milwaukee WI) dissolved in 5 ml of dry chloroform was added slowly over a period of 20 minutes at 4°C. and the reaction mixture was allowed to warm to room temperature with stirring. After 20 hours the reaction was stopped with the addition of 8 ml of distilled water, and 38 ml of chloroform/methanol/0.5N HCl (1/2/0.8 by volume) was added. The phases were separated by the addition of 10 ml of chloroform and 8 ml of 0.5N HCl. The organic phase containing the required compound was further washed with 0.5N sodium bicarbonate. The lower chloroform phase was dried over anhydrous sodium sulfate and evaporated under vacuum. The compound was crystallized from chloroform/acetone at -20°C. Further purification was obtained by silicic acid column chromatography, and 145 mg of pure 5'-palmitoyl(3'-azido, 3'-deoxy)thymidine was obtained (yield 15.3%). Elemental analysis: Predicted C

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61.59, H 8.5, N 13.8 and O 15.8; Found C 60.74, H 8.6, N 13.5 and O 17.9. Rf on silica gel G thin layer chromatography plates: 0.92 (chloroform/methanol/ammonia/water, 70/30/1/1); 0.83 5 (hexane/ethylether/acetic acid, 80/20/1) and 0.86 (chloroform/acetone, 94/6), m.p. 77-80°C. UV_{max} 265.

Efficacy of PalmitoylAZT in HIV-Infected HT4-6C Cells

PalmitoylAZT was incorporated into liposomes as noted in 10 Example 8 and incubated with LAV-1 infected HT4-6C cells as noted in Examples 10 and 11. 0.8 uM palmitoylAZT inhibited plaque formation by 25% (134 plaques versus 176 in the untreated control).

15

It should be apparent from the foregoing that other nucleoside analogues and phospholipid derivatives thereof can be substituted in the Examples to obtain similar results. AZT-monophosphate or other antiviral nucleoside phosphate may also be contained in the aqueous compartments of the liposome. The molar percentage of the lipid antiviral nucleoside may vary from 0.1 to 100% of the total lipid mixture. Furthermore, mixtures of antiviral nucleoside lipids may be used in constructing the 20 liposomes for therapy of viral diseases. It should be further emphasized that the present invention is not limited to the use of any particular antiviral nucleoside 25 analogue; rather, the beneficial results of the present invention flow from the formation of liposomes from the lipid derivatives of these materials. Thus, regardless of 30 whether an antiviral nucleoside is presently known, or whether it becomes known in the future, the methods of forming the presently-contemplated lipid derivatives therefrom are based on established chemical techniques, as 35 will be apparent to those of skill in the art, and their incorporation into liposomes is broadly enabled by the

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preceding disclosure. It should be emphasized again that the present syntheses are broadly applicable to formation of compounds from essentially all nucleoside analogues for use in the practice of the present invention.

5 Accordingly, the invention may be embodied in other specific forms without departing from its spirit or essential characteristics. The described embodiments are to be considered in all respects only as illustrative and not restrictive, and the scope of the invention is, 10 therefore, indicated by the appended claims rather than by the foregoing description. All modifications which come within the meaning and range of the lawful equivalency of the claims are to be embraced with their scope.

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WHAT IS CLAIMED IS:

1. A compound having antiviral properties,
5 comprising:

a nucleoside analogue having a base portion comprising a purine or pyrimidine or analogue thereof, and a sugar portion comprising a pentose residue, wherein at least one said portion is a non-naturally occurring nucleoside component; and

10 a lipid moiety linked to said pentose residue; with the proviso that said compound is in the form of a liposome when said pentose residue is arabinofuranose and said base portion is cytosine or adenine.

15 2. The compound of Claim 1, wherein said non-naturally occurring nucleoside component is an analogue of a naturally occurring base or pentose by virtue of substitution, deletion, or replacement.

20 3. A compound according to Claim 1, wherein said pentose residue is a 2',3'-dideoxy, 2',3'-didehydro, azido or halo derivative of ribose, or an acyclic hydroxylated fragment of ribose.

25 4. A compound according to Claim 3, wherein said pentose residue is a 2',3'-dideoxyribose, and said nucleoside analogue is 2',3'-dideoxycytidine; 2',3'-dideoxythymidine; 2',3'-dideoxyguanosine; 2',3'-dideoxyadenosine; 2',3'-dideoxyinosine; or 2,6-diaminopurine, 2',3'-dideoxyriboside.

30 5. A compound according to Claim 3, wherein said pentose residue is a 2',3'-didehydroribose and said nucleoside is 2',3'-didehydrothymidine; 2',3'-didehydrocytidine carbocyclic; or 2',3'-didehydroguanosine.

35 6. A compound according to Claim 3, wherein said pentose residue is an azido derivative of ribose, and said nucleoside is 3'-azido-3'-deoxythymidine;

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3'-azido-3'-deoxyguanosine; or 2,6-diaminopurine-3'-azido-2',3'dideoxyriboside.

7. A compound according to Claim 3, wherein said pentose residue is a halo derivative of ribose and said nucleoside is 3'-fluoro-3'-deoxythymidine; 3'-fluoro-2',3'-dideoxyguanosine; 2',3'-dideoxy-2'-fluoro-ara-adenosine; or 2,6-diaminopurine-3'-fluoro-2',3'-dideoxyriboside.

8. A compound according to Claim 3, wherein said pentose residue is an acyclic hydroxylated fragment of ribose, and said nucleoside is 9-(4,-hydroxy-1',2'-butadienyl) adenine, 3-(4,-hydroxy-1',2'-butadienyl) cytosine, 9-(2-phosphonylmethoxyethyl)adenine or 3-phosphonomethoxyethyl, 2,6-diaminopurine.

9. The compound of Claim 1, wherein said nucleoside analogue is acyclovir, gancyclovir, 1-(2'-deoxy-2'-fluoro-1- β -D-arabinofuranosyl)-5-iodocytosine (FIAC) or 1(2'-deoxy-2'-fluoro-1- β -D-arabinofuranosyl)-5-iodouracil (FIAU).

10. The compound of Claim 1, wherein said nucleoside analogue is 2-chlorodeoxyadenosine.

11. The compound of Claim 1, wherein said nucleoside analogue is a 3'-azido-2',3'dideoxypyrimidine selected from the group consisting of AzddClU, AzddMeC, AzddMeC N4-OH, AzddMeC N4Me, AzddEtU, AzddU, AzddC, AzddFC, AzddBrU, and AzddIU.

12. The compound of Claim 1, wherein said nucleoside analogue is a 3'-halopyrimidine dideoxynucleoside selected from the group consisting of 3'-FddC;U, 3'-FddU, 3'-Fddt, 3'-FddBrU, and 3'-FddEtU.

13. The compound of Claim 1, wherein said nucleoside analogue is a 2',3'-didehydro-2',3'-dideoxynucleoside selected from the group consisting of D4T, D4C, D4MeC, and D4A.

14. The compound of Claim 1, wherein said nucleoside

is a 2',3'-unsubstituted dideoxypyrimidine nucleoside selected from the group consisting of 5-F-ddC, ddC and ddT.

15. The compound of Claim 1, wherein said nucleoside is a 2',3'-unsubstituted dideoxypurine nucleoside selected 5 from the group consisting of ddA, ddDAPR, ddG, ddI, and ddMeA.

16. The compound of Claim 1, wherein said nucleoside is a sugar-substituted dideoxypurine nucleoside selected from the group consisting of 3-N₃ddDAPR, 3-N₃ddG, 3-
10 FddDAPR, 3-FddG, 3-FddaraA, and 3-FddA.

17. A compound according to any one of Claims 1 through 16, further comprising a monophosphate, diphosphate, or triphosphate linking group between the 5' position of said pentose residue and said lipid moiety.

18. Phosphatidyl(3'-azido-3'-deoxy)thymidine (pAZT).

19. Phosphatidyl(2',3'-dideoxy)cytidine (pddC).

20. Phosphatidyl(2',3'-dideoxy)thymidine (pddT).

21. (3'-azido-3'-deoxy)thymidine diphosphate diglyceride (AZTdpdg).

22. Phosphatidylacyclovir (pACV).

23. 1-O-stearoylglycero-rac-3-phospho-5'-(3'-azido, 3'-deoxy)thymidine.

24. A compound according to any one of Claims 1 through 16, further comprising an aliphatic bridge 25 comprising two functional groups and having from 0 to 10 carbon atoms between said functional groups, said bridge joining said lipid and said pentose residue.

25. A compound according to any one of Claims 1 through 16, wherein said lipid moiety is a fatty acid.

30. A compound according to any one of Claims 1 through 16, wherein said lipid moiety is a monoacylglycerol or a diacylglycerol.

35. A compound according to any one of Claims 1 through 16, wherein said lipid moiety is a phosphatidic acid.

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28. A compound according to Claim 1, wherein said lipid is a phospholipid having a head group comprising a sugar or a polyhydric alcohol.

29. A compound according to Claim 27, wherein said 5 lipid moiety comprises bis(diacylglycero)phosphate.

30. A compound according to Claim 27, wherein said lipid moiety comprises a diphosphatidylglycerol.

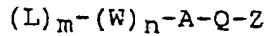
31. A compound according to Claims 1 through 16, wherein said lipid moiety is a D,L-2,3-diacyloxypropyl-10 (dimethyl)-beta-hydroxyethyl ammonium group.

32. A compound according to Claim 1, wherein said lipid moiety comprises from 1 to 4 fatty acid moieties, each said moiety comprising from 2 to 24 carbon atoms.

33. A compound according to Claim 27, wherein at 15 least one fatty acid moiety of said lipid moiety is unsaturated, and has from 1 to 6 double bonds.

34. A compound according to Claim 1, comprising 1,2-diacylglycerophospho-5'-(2',3'-dideoxy)thymidine.

35. A compound according to Claim 1, having the 20 formula:



wherein

Z is the base portion of said nucleoside analogue;

Q is the pentose residue;

25 A is O, C, or S;

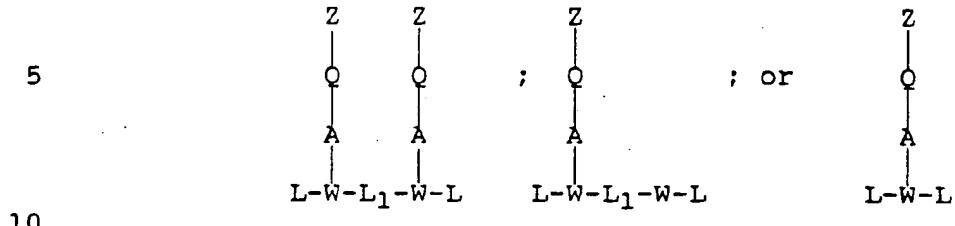
W is phosphate;

n = 0 to 3;

and L is a lipid moiety, wherein m = 1 to 5; and wherein each L is linked directly to a W except when n=0, in which 30 case each L is linked directly to A.

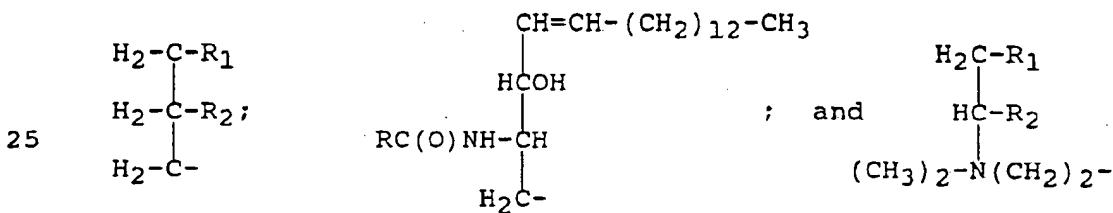
-73-

36. A compound according to Claim 1, having the formula:



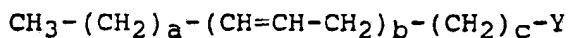
wherein Z is the substituted or unsubstituted purine or pyrimidine group of said nucleoside analogue,
Q is the pentose residue;
A is O, C, or S;
15 W is phosphate;
L₁ is (CH₂-CHOH-CH₂); and
L is a lipid moiety.

37. A compound according to Claim 35 or 36, wherein
each L is independently selected from the group consisting
20 of R;



wherein R, R₁ and R₂ are independently C₁ to C₂₄ aliphatic groups.

38. A compound according to Claim 37, wherein R, R₁ and R₂ independently have from 0 to 6 sites of unsaturation, and have the structure



35 wherein the sum of a and c is from 1 to 23; and b is 0 to 6; and wherein Y is $C(O)O-$, $C-O-$, $C=C-O-$, $C(O)S-$, $C-S-$, or $C=C-S-$.

39. A compound according to any one of Claims 35-38, wherein said pentose residue comprises ribose, 40 dideoxyribose, didehydroribose, or an azido or halo substituted ribose, attached at the 9 position of said purine or at the 1 position of said pyrimidine.

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40. A liposome formed at least in part from the compound of any one of Claims 1-39.

41. A method for synthesizing a lipid derivative of an antiviral nucleoside, comprising the step of reacting 5 an antiviral nucleoside, having a ribose hydroxyl group, with a phospholipid in the presence of a coupling reagent whereby said nucleoside is joined to said phospholipid by a phosphate bond at the position of said ribose hydroxyl group, to form a compound according to any one of Claims 10 1-38.

42. The method of Claim 41, wherein the phospholipid is a diacyl phosphate.

43. The method of Claim 41, wherein said phospholipid is a phosphatidic acid.

44. The method of Claim 41, wherein said phospholipid is a ceramide.

45. A method of synthesizing a lipid derivative of an antiviral nucleoside, comprising the steps of:

reacting an antiviral nucleoside monophosphate 20 with a reagent HL, wherein L represents a leaving group, to form a nucleoside $\text{PO}_4\text{-L}$;

reacting said nucleoside $\text{PO}_4\text{-L}$ with a phosphatidic acid to join said acid to said nucleoside through a pyrophosphate bond.

46. The method of Claim 45, wherein said nucleoside monophosphate is AZT 5'-monophosphate.

47. A method of synthesizing a glyceride derivative of a nucleoside analogue, comprising the step of joining a monoglyceride or diglyceride and an antiviral nucleoside 30 monophosphate with a coupling agent in the presence of a basic catalyst.

48. The method of Claim 47, wherein said glyceride is 1-O-stearoylglycerol and said nucleoside is AZT monophosphate.

49. A method according to any one of Claims 41, 45,

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or 47, wherein said nucleoside analogue comprises an adenine or cytidine moiety, comprising the steps of:

blocking reactive amino groups of said moiety prior to the coupling reaction; and

5 deblocking said groups after said nucleoside analogue is joined to a lipid.

50. A method for treating a viral infection in a mammal, comprising the step of administering an effective amount of a compound according to any one of Claims 1-39.

10 51. A method according to Claim 50, wherein said viral infection is a herpes simplex infection in a human, and said compound is phosphatidylacyclovir.

52. A method according to Claim 50, wherein said mammal is a human and said virus is HIV retrovirus.

15 53. A method according to Claim 52, wherein said compound is 5'-palmitoylAZT.

54. A method according to Claim 52, wherein said retrovirus is a strain of HIV that has developed resistance to a nucleoside analogue.

20 55. A method for prolonging the antiviral effect of a nucleoside analogue in a mammal, comprising administering the nucleoside analogue to the mammal in the form of the compound of any one of Claims 1-39.

56. The method of Claim 54, wherein said method further includes avoiding or overcoming resistance of the retrovirus to nucleoside analogues through administering said analogue in the form of said compound.

25 57. A method for preparing a suspension of liposomes for use in treating viral infections in a mammal, comprising:

providing a lipophilic antiviral agent comprising at least one lipid species attached to a nucleoside analogue;

30 combining the lipophilic antiviral agent and a pharmacologically acceptable aqueous solvent to form a mixture; and

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forming liposomes from the lipophilic antiviral agent.

58. Use of the compound of any one of Claims 1-39 in the preparation of a medicament for treatment of a human viral infection.

59. Use of a compound as claimed in any one of claims 1-39 for the treatment of a human viral infection.

60. Use of a compound as claimed in any one of claims 1-39 for the treatment of HIV infection in a human.

10 61. A compound as claimed in any one of claims 1-39 for use in the treatment of a human viral infection.

62. A compound as claimed in any one of claims 1-39 for use in the treatment of HIV infection in a human.

15 63. A pharmaceutical composition comprising a compound as claimed in any one of claims 1-39 and a pharmaceutically acceptable carrier.

64. A pharmaceutical composition comprising a compound as claimed in any one of claims 1-39 and at least one other antiviral compound.

20 65. Use of a composition as claimed in claim 63 or 64 for the treatment of a human viral infection.

66. A composition as claimed in claim 63 or 64 for use in the treatment of a human viral infection.

H533-1: EFFECT OF LIPONUCLEOTIDES ON p24
PRODUCTION BY CEM CELLS

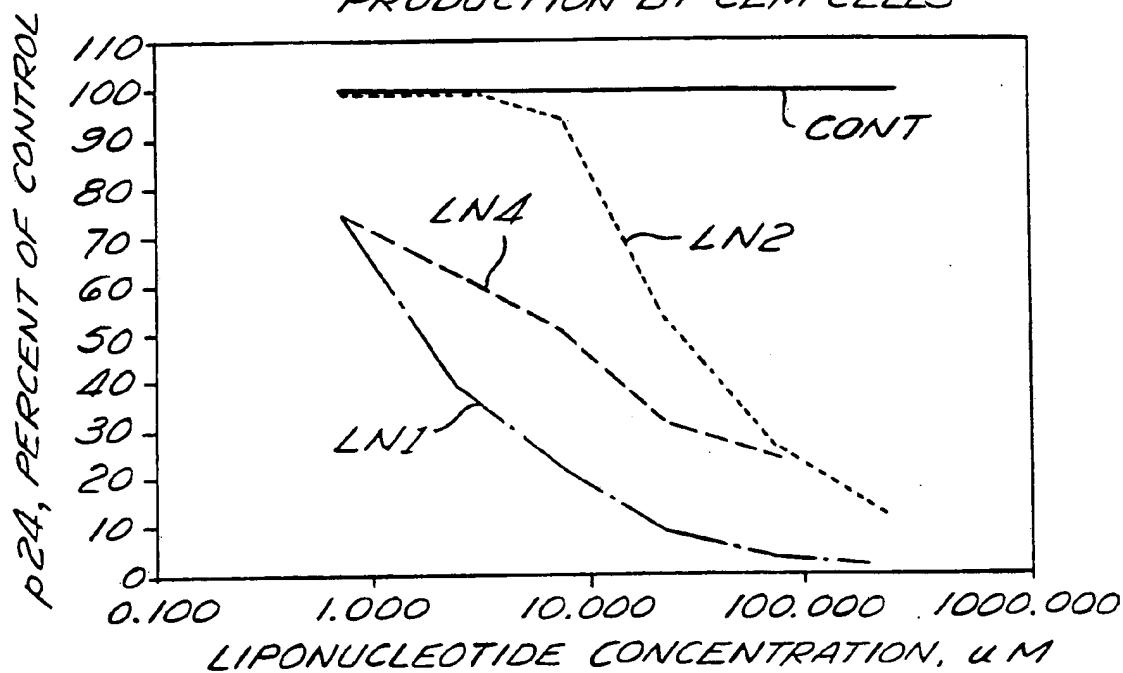


Fig. 1

H747-1Q: EFFECT OF AVN AND LIPONUCLEOTIDES
ON p24 PRODUCTION BY CEM WT CELLS

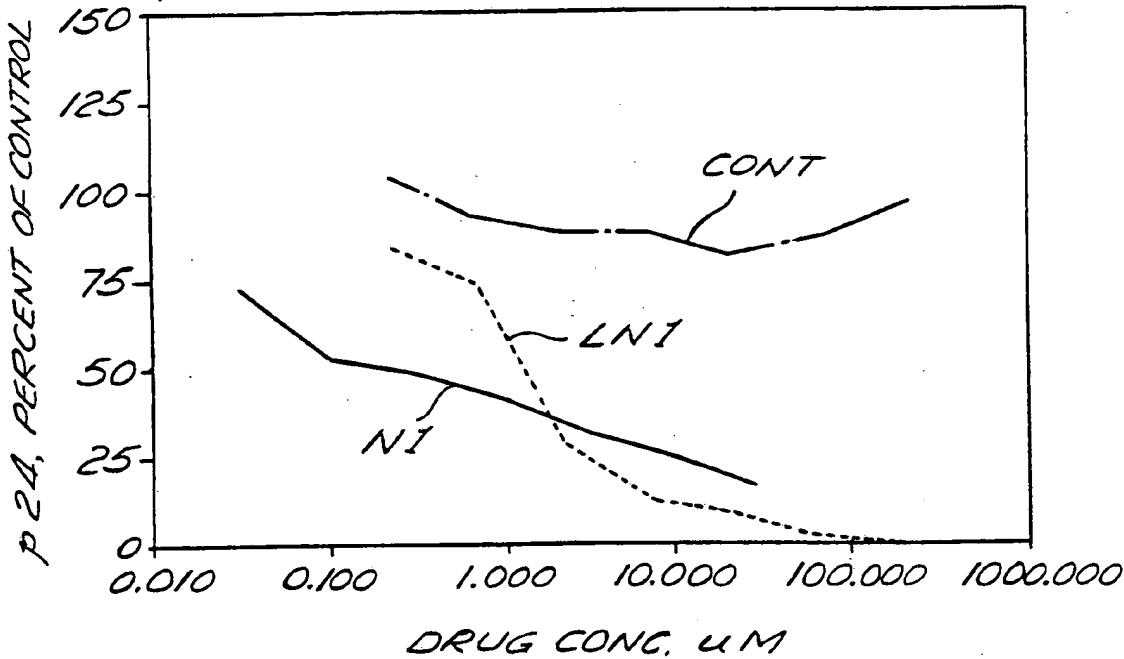


Fig. 2

H747-1B: EFFECT OF AVN AND LIPONUCLEOTIDES
ON p24 PRODUCTION BY CEM WT CELLS

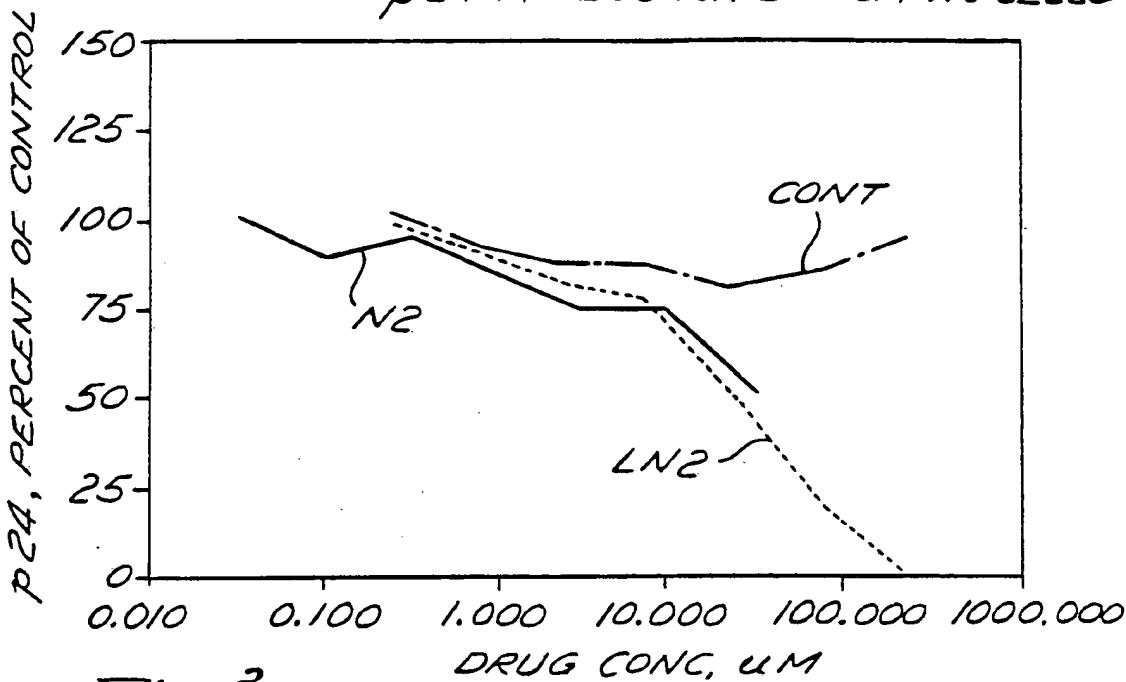


Fig. 3

H637-1B: EFFECT OF AVN AND LIPONUCLEOTIDES
ON p24 PRODUCTION BY CEM TK - CELLS IN VITRO

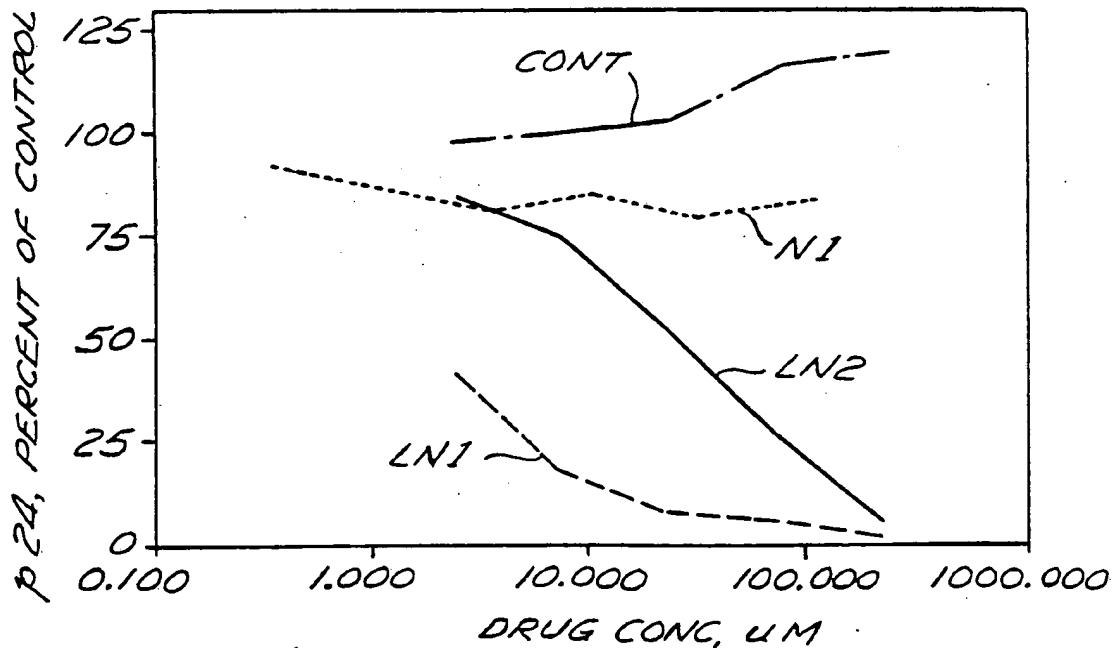


Fig. 4

H805-1: EFFECT OF AVN AND LIPONUCLEOTIDES ON p24 PRODUCTION BY CEM WT CELLS IN VITRO

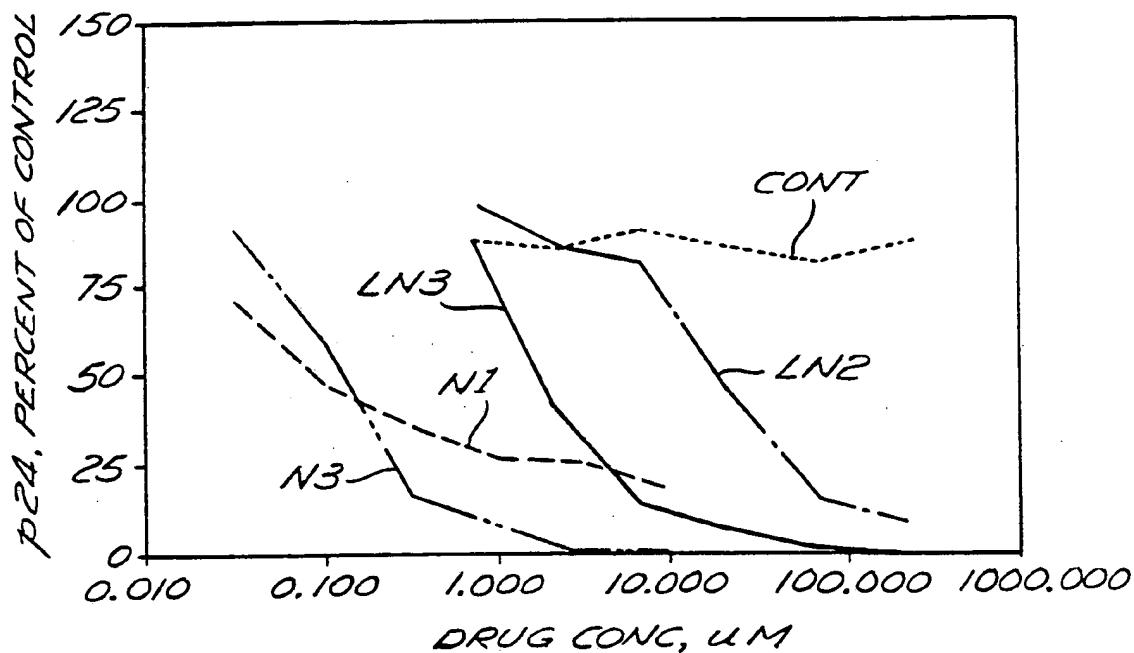


Fig. 5

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/02909

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

INT: CL: C07H 15/12; C07H 17/00

U.S. CL: 536/27, 28, 29; 425/450; 514/49, 51

II. FIELDS SEARCHED

Minimum Documentation Searched⁷

Classification System	Classification Symbols
U.S. CL.	536/27, 28, 29 424/450 514/12, 49, 51, 159, 808

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched⁸III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	1 Relevant to Claim No. ¹³
X	US, A, 4,291,024 (TURCOTTE) 22 SEPTEMBER	1-66
Y	1981, SEE COLUMNS 2-6, CLAIMS 1 AND 8.	1-66
Y	US, A, 4,471,113 (MacCROSS) 11 SEPTEMBER 1984, SEE COLUMN 3, lines 46-COLUMN 4, LINES 37.	1-66
A	US, A, 4,622,392 (HONE) 11 NOVEMBER 1986, SEE COLUMNS 2-6.	1-66
A	US, A, 4,692,433 (HOSTETLER) 08 SEPTEMBER 1987, SEE COLUMN 2.	1-66
Y	US, A, 4,283,394 (WEST) 11 AUGUST 1981, SEE COLUMNS 2-3.	1-66
A	G. L. SCHERPHOF, "LIPOSOMES IN BIOLOGY AND MEDICINE," IN <u>LIPIDS AND BIOMEMBRANES, PAST, PRESENT, AND FUTURE</u> , PUBLISHED 1986 BY ELSEVIER NORTH HOLLAND (AMSTERDAM), P. 113-136.	1-66

* Special categories of cited documents:¹⁰

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

16 AUGUST 1989

Date of Mailing of this International Search Report

30 OCT 1989

International Searching Authority

ISA/US

Signature of Authorized Officer

GARY L. KUNZ

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	G. POST ET AL., "THE CHALLENGE OF LIPOSOME TARGETING IN VIVO, " IN <u>LIPOSOME TECHNOLOGY</u> , VOLUME III, PUBLISHED 1984, BY CRC PRESS (BOCA RATON), P. 1-28.	1-66
A	MARC J. OSTRO, "LIPOSOMES," IN <u>SCIENTIFIC AMERICAN</u> , VOLUME 256, PUBLISHED 1987, 103-111.	1-66
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE, VOLUME 75, NO. 6, ISSUED 1978, JUNE, SEE ABSTRACT.	1-66
A	THE JOURNAL OF BIOLOGICAL CHEMISTRY, VOLUME 255, NO. 17, ISSUED 1980 SEPTEMBER 10, (WASHINGTON), A HUANG ET AL., "MONOCLONAL ANTIBODY COVALENTLY COUPLED WITH FATTY ACID," SEE PAGE 8015-8018.	1-66
A	<u>CANCER RESEARCH</u> , VOLUME 37, ISSUED 1977 JUNE, D. H. W. HA, "PHARMACOLOGY OF 5'-ESTERS OF 1-B-D-ARABINOSYLCYTOSINE," SEE P. 1640-1643.	53
A	<u>CANCER RESEARCH</u> , VOLUME 317, ISSUED 1987 JULY, N. A. FISCHL ET AL., "THE EFFICACY OF AZIDOTHYMIDINE (AZT) IN THE TREATMENT OF PATIENTS WITH AIDS AND AIDS RELATED COMPLEX," P. 185-191	1-66
A	JOURNAL OF EXPERIMENTAL MEDICINE, VOLUME 166 ISSUED 1987 OCTOBER, D.D. RICHMAN ET AL., "FAILURE OF DIDEOXYNUCLEOSIDES TO INHIBIT HUMAN IMMUNODEFICIENCY VIRUS REPLICATION IN CULTURED HUMAN MACROPHAGES," P. 1144-1149.	1-66

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. Claim numbers _____, because they relate to subject matter¹² not required to be searched by this Authority, namely:

2. Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out¹³, specifically:

3. Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

SEE ATTACHMENT.

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. **TELEPHONE PRACTICE**

2. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

The additional search fees were accompanied by applicant's protest.

No protest accompanied the payment of additional search fees.